

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

EDITORIAL BOARD

KEIZO KODAMA

TOMIHIDE SHIMIZU

FOKURO SODA

PUBLISHED QUARTERLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

PRINTED IN JAPAN

The JOURNAL OF BIOCHEMISTRY, founded in 1922 by Prof. emeritus S. Kakiuchi, was discontinued in 1944, Vol. 36, after the duration of 22 years. Thereafter, the NIPPON SEIKAGAKU-KAISHI or the JOURNAL OF JAPANESE BIOCHEMICAL SOCIETY, both in Japanese, has been designed for the publication of original investigations and some reviews.

THE JOURNAL OF BIOCHEMISTRY, republished in 1950, is conducted by the Japanese Biochemical Society, and edited by three editors elected in the General Meeting of the Society, in April 1950.

The new issue, in 1950, following the serial number of the discontinued Journal will be Vol. 37.

All papers for publication should be addressed to Prof. K. Kodama, Institute of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.

This Journal is issued quarterly, one volume per year.

SUBSCRIPTIONS. U. S. \$7.00 per volume, postage inclusive. Remittance should be made by check or draft, payable to the Japanese Biochemical Society, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.

The back issues are available for sale, from Vol. 1-36, U. S. \$5.50 per volume, postage inclusive.

INHIBITORY EFFECT OF CAFFEINE AND METHYLENE BLUE ON AMINE OXIDASE.*

By

YUKITO OOTA.

*(From the Biological Institute, Faculty of Science,
Nagoya University, Nagoya.)*

(Received for publication, April 22, 1950)

Mann and Quastel (1) has found that benzedrine (β -phenylisopropylamine) can protect brain tissue against the anoxaemia induced by tyramine. They explain this as follows: benzedrine, by virtue of its structural similarity to the substrate amines of amine oxidase, including tyramine, is capable of inhibiting the oxidase in a competitive manner, thus tyramine is prevented from passing into its oxidation product, viz., an aldehyde, which can exert an inhibitory action on the tissue respiration. In addition, these authors suggest a possibility that this effect may be intimately connected with the central nerve stimulating effect of benzedrine. In the previous experiments (2), the author has extended the observations of Mann and Quastel, employing more than thirty benzedrine derivatives, where fairly good parallelism between the ability of oxidase inhibition and that of central nerve stimulation was demonstrated, favoring their view above-mentioned. The present paper communicates a chance observation that quite the same effect as that of benzedrine is also brought about by caffeine, viz., exertion of a competitive inhibition upon amine oxidase and thus removal of the tyramine-poisoning of brain respiration. No clear cut result, however, was obtained as to the problem whether to this effect the pharmacological action of this stimulant might be ascribed. Regarding the unexpect-

* Aided by a grant from the Department of Education.

ed affinity of caffeine, which has apparently no structural similarity to the substrate amines, to the active center of amine oxidase, specific affinity of N-methyl group to the oxidase was induced. Wellknown toxicity of methylene blue on the oxidase was also interpreted as being due to the very effect of the groups in the pigment molecule.

EXPERIMENTAL

Preparation of Amine Oxidase (cf., Richter (3))—Guinea-pig liver is cut finely with scissors, minced with quartz-sand. The brei is mixed with three times the volume of phosphate buffer solution ($M/30$; pH 7.0), and stirred for about 20 minutes. After centrifugation, the supernatant solution is dialyzed with a cellophane sack for about 5 hours against running tap water. The dialysate is used as enzyme preparation. The spontaneous oxygen uptake of the preparation is practically negligible. It will keep, if stored in an ice-chest, for at least 5 days with little loss of activity.

Preparation of Brain Brei—Guinea-pig is killed by decapitation, and the brain is instantly removed. It is washed with distilled water, and veins are picked away with a pincette as sufficiently as possible. The tissue is minced in a mortar and phosphate buffer solution ($M/20$; pH 7.6) of three times its weight is added. The mixture is stirred well and squeezed through silk stuff. Milky white brei thus obtained is employed in the experiment.

The rate of oxygen uptake of the enzyme preparation (oxidation of tyramine added as substrate) as well as that of the brain brei (endogenous respiration without any addition of substrate compound) are determined manometrically using Warburg apparatus. Gas rooms of the vessels are filled with air; all experiments are carried out at 30°.

RESULTS

Effect of Caffeine on Tyramine-Inhibited Brain Respiration—

Table I shows exactly the same recovering effect of caffeine on tyramine-inhibited brain respiration as that of benzedrine. Previously for 2 hours before the start of experiment the brain brei had been treated with *M/500* of tyramine, whose oxidation was meanwhile practically completed, thus resulting the maximum depression (about

TABLE I

Effect of Tyramine and Caffeine on Brain Respiration.

Tyramine-HCl (<i>M/500</i>)	—	+	—	+	
Caffeine (<i>M/50</i>)	—	—	+	+	
O ₂ -uptake (μl/60 min.)	58.2	26.1	47.5	41.8	

1.5 ml., brain brei in phosphate buffer (*M/20*; pH 7.6); 0.3 ml., tyramine-HCl (*M/50*); 0.6 ml., caffeine (*M/10*); With water made up to 3.0 ml. Both tyramine and caffeine were added 2 hours prior to the commencement of the measurement. Warburg apparatus; Air, 30°.

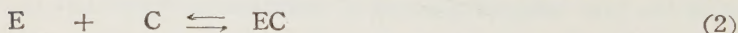
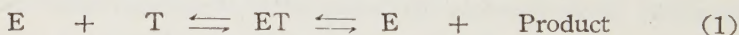
55% inhibition) in the rate of oxygen uptake in the tissue concerned. Whereas simultaneous supply of caffeine (*M/50*) relieved this depression by half.

Caffeine in itself, however, was proved to be poisonous to brain respiration. As is shown in the table, *M/50* of caffeine when added alone to the brei induced about 20% inhibition in the oxygen uptake. It is remembered that benzedrine itself is also an inhibitor of brain respiration, thus, *M/700* of benzedrine exerts inhibition on brain respiration to the extent of about 25% (1). And the same amount of benzedrine has to be added to brain preparation so as to recover by half the oxygen uptake depressed by *M/50* of tyramine (1). In these respects, therefore, caffeine is about one fifteenth as effective as benzedrine.

Benzedrine is unable to act as an antidote against poisonous action of narcotics on the tissue respiration (1). It was also the

case with caffeine. For example, chloretone (0.03%), sodium salt of veronal (0.1%), and narphine (0.01%) depressed the oxygen uptake of the brain brei by 21.6, 9.3, and 24.5%, respectively, while the simultaneous presence of caffeine ($M/50$) increased the inhibition as much as 35.0, 26.3, and 29.5%, respectively. Except the case of narphine, in the coexistence of caffeine and narcotic approximately additive inhibition was resulted, indicating little mutual interference between the inhibitory mechanisms of these reagents.

Inhibition of Amine Oxidase by Caffeine—Recovering phenomenon in brain respiration from tyramine-inhibition suggests that there should occur some disturbance in the process of tyramine oxidation, or in the activity of amine oxidase. In fact, it was found that caffeine did inhibit amine oxidase. The effect of caffeine ($M/100$) on the initial rate of oxidation of various amounts of tyramine was measured, and as the results summarized in Table II show, the magnitude of inhibition caused by caffeine was in inverse proportion to the amount of substrate supplied, thus, a competitive nature of the inhibition was indicated. Suppose that here rule essentially the same mode of inhibition as that known in benzedrine, i. e. reversible formation of inert complex between caffeine molecule (C) and the enzyme (E), then the tyramine (T) oxidation by amine oxidase in the presence of caffeine will be written as:



For the magnitude of inhibition defined as: $I = 1 - V'/V$, we can formulate

$$I = \frac{[C]}{K \left(1 + \frac{[T]}{K_m} \right) + [C]} \quad (3)$$

where signify V and V' , initial rates of oxygen uptake due to the tyramine oxidation by amine oxidase in the absence and in the presence of caffeine, respectively; K_m , Michaelis constant, the value

of which has been estimated at $10^{-3.6}$ mole/lit. at pH 7.6 and 30° (tyramine as substrate) (2); K , dissociation constant (mole/lit.) of the enzyme-caffeine complex; $[C]$ and $[T]$, concentrations of caffeine and tyramine, respectively.

As shown in Table II, all K values obtained under the conditions employed have gathered in the vicinity of the mean value: $10^{-2.7}$ mole/lit. hence we may conclude probable validity of the above presumed mode of inhibition of the enzyme by caffeine.

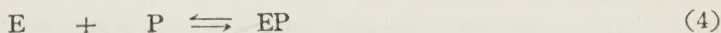
TABLE II.

Inhibition of Tyramine Oxidation by Caffeine ($10^{-2.0}$ M)

Tyramine-HCl (M)	$10^{-2.7}$	$10^{-2.4}$	$10^{-2.0}$	$10^{-1.7}$
Inhibition, I (%)	38.6	29.1	12.2	4.1
$-\log K$	2.8	2.8	2.7	2.5

1.5 ml., oxidase preparation: 0.3 ml., tyramine-HCl; 0.3 ml., caffeine ($M/10$), 0.3 ml., phosphate buffer ($M/5$; pH 7.6); 0.6 ml., water. Tyramine and caffeine were added simultaneously at time zero, and oxygen uptake during the initial 5 min. were read. Warburg apparatus; Air, 30° .

Here is another fact favorable to our postulation. If caffeine and philopon (D β -phenyl-isopropylmethylamine) (P) are simultaneously added to amine oxidase, with the reactions 1 and 2, the third reaction 4:



must be accompanied. Then the magnitude of inhibition will be given by the equation:

$$I = \frac{K_m \left(\frac{[C]}{K} + \frac{[P]}{K'} \right)}{K_m \left(1 + \frac{[C]}{K} + \frac{[P]}{K'} \right) + [T]} \quad (5)$$

where the dissociation constant of the complex EP (K') has been found to be $10^{-4.2}$ mole/lit. at pH 7.6 and 30° (2), and the values of both K (dissociation constant of the complex EC) and K_m were already mentioned above. Under the conditions: $[C]=10^{-2.0}$, $[P]=10^{-3.0}$, and $[T]=10^{-2.7}$, the oxidase activity was observed to be depressed by 71.4% (pH 7.6; 30°), while theoretical value of inhibition calculated by the equation 5 was 70%, with which the experimental value was in good agreement.

Practically no effect of previous treatment of amine oxidase with caffeine was detectable. Prior to the addition of tyramine, $M/100$ of caffeine was incubated with the enzyme preparation for 0, 30, and 60 minutes, respectively, and 39.2, 39.0, and 39.0% inhibition in tyramine oxidation were resulted correspondingly.

Effect of Purine and Pyrimidine Compounds on Amine Oxidase—

Several purine and pyrimidine compounds with regard to their influences on amine oxidase were studied (Table III). Scarcely soluble purines, shown with asterisk in the table, were previously solved in sodium hydroxide solution, and before use neutralized with hydrochloric acid to the point just before precipitate appeared. As it was, however, impossible to preclude these compounds from precipitating to some extent in the medium employed (pH 7.6) during experimental period, the results could not stand quantitative comparison. In these compounds, inhibitory effect on the oxidase and stimulating action on the central nerve do not always run parallel. Besides caffeine, theobromine, and theophylline (methyl-purines) which are known as purine-stimulants, guanine (amino-purine) which is believed to have no stimulant action, and, moreover, even veronal, wellknown narcotic, also reveal themselves to be more or less inhibitory to the oxidase. On the contrary, uric acid as well as xanthine (both hydroxypurines) and alloxan compounds without stimulating effect on the central nerve, have no inhibitory, but rather promoting action on the oxidase activity. Hence, with the inhibitory action in question purine or pyrimidine skeleton

itself may have little to do, while the very existence of N-methyl or amino group in the molecule should probably be of significance.

TABLE III.

*Effects of Purine and Pyrimidine Compounds (M/100)
on Amine Oxidase*

	Inhibition	Awakening Action
Caffeine	++	++
Theobromine*	++	++
Theophylline	+	+
Guanine-HCl*	+++	—
Uric Acid*	—	—
Xanthine*	—	—
Alloxan	—	—
Veronal-Soda	+	—

1.5 ml., oxidase preparation; 0.3 ml., tyramine-HCl (*M*/50); 0.3 ml., purine or pyrimidine derivative (*M*/10); 0.3 ml., phosphate buffer solution (*M*/5; pH 7.6); 0.6 ml., water. The rates of initial O₂-uptake (μ l/5 min.) were measured manometrically, at 30°. The enzyme preparation contained considerable amounts of uricase, hence when uric acid was examined, blank tests were performed. No xanthine oxidase was detectable in our preparation.

Effect of Methylene Blue on Amine Oxidase—Since amine oxidase attacks the substrate deaminatively, it seems enough natural that the enzyme may have general affinity to amino group. It is, however, curious that the oxidase is also able to combine with N-methyl group, hence it becomes our interest to see if any compound apparently without any structural similarity to the substrate amines but provided with N-methyl groups in its molecule can exert competitive inhibition on the oxidase activity. Out of several favorable facts obtained the results with methylene blue will be reported in some detail here (Table IV).

TABLE IV.

Inhibition of Amine Oxidase by Methylene Blue (M/375,000)

Tyramine (M)	10 ^{-3.1}	10 ^{-2.7}	10 ^{-2.4}	10 ^{-2.1}
Inhibition (%)	63.2	42.4	31.8	19.6
-log K	6.5	6.4	6.5	6.5

1.25 ml., amine oxidase preparation; 0.5 ml., tyramine-HCl; 0.1 ml., methylene blue (M/15,000); 0.25 ml., phosphate buffer (M/5; pH 7.6); 0.4ml., water; Warburg apparatus; Air, 30°C, 5 min.

Methylene blue, which has two dimethylamino groups in its molecule, exerted competitive inhibition on amine oxidase in quite the same way as that disclosed about benzedrine as well as caffeine. The dissociation constant of the enzyme-pigment complex was found to be $K=10^{-6.5}$ mole/lit.; the most prominent affinity to amine oxidase ever known (So far, $K=10^{-4.7}$ for β -3:4-methylenedihydroxyphenyl-isopropylamine had been the greatest (2)).

Thionine, methylene blue derivative without methyl group, did also inhibit amine oxidase, but far less effective than methylene blue, and, moreover, the mode of inhibition appeared to be non-competitive. It will be noteworthy that the effectiveness of methylene blue disappears sooner or later with time of storage of the pigment in aqueous state. This probably corresponds to the known fact that this somewhat autoxidizable pigment loses its methyl groups by degrees, thus converting itself into an inert azur body.

Other compounds, which have N-methyl groups, such as crystal violet dimethyl-p-phenylenediamine, were investigated and were found to be also inhibitory to the oxidase. Corresponding amino compounds without methyl group: fuchsine, *p*-phenylenediamine, appeared to be not effective. In these cases reproducible results were not obtained; the reason is yet obscure.

DISCUSSION

Caffeine which *per se* affects depressingly on brain respiration, is able to release the oxygen uptake in brain from the tyramine-inhibition. This reminds us of the analogous situation of benzedrine. The effectiveness of caffeine, however, is only one-fifteenth as much as benzedrine. The poisonous action of caffeine on tissue respiration may probably be attributed to the existence of non-polar (methyl) groups in its molecule, as is the case with narcotics (cf., Sherif (4), who has reported an unspecific inhibitory effect of caffeine on the dehydrogenase systems in sciatic nerve). In this respect, the fact that the inhibitory action of caffeine on brain respiration can take place almost without any interference with that of narcotic such as chloretone or veronal, is rather an enigma, which should be clarified in future.

Although it is yet doubtful if there can exist any relation between the recovering effect of caffeine on the tyramine-inhibited brain respiration and its pharmacological action, it will still be premature to deny all the possibilities. At least every purine compounds which have stimulating action on central nervous tissue, viz., not only caffeine but theobromine and theophylline, are capable of inhibiting amine oxidase, thus they are expected to have recovering action on brain respiration from the tyramine-inhibition. And, if the pharmacological action of benzedrine will be attributed to its protecting action on brain respiration against the tyramine-inhibition, the following fact may suggest that the same thing will also be valid for caffeine: As we have pointed out, regarding the recovering activity on tyramine-inhibited brain respiration, benzedrine is fifteen times as effective as caffeine, and the ratio is approximately equal to that of the usual doses (*per os*) of these two stimulants, i. e., 0.05–0.5 g. a day (caffeine) to 0.003–0.006 g. a day (benzedrine).

As to the affinity of caffeine to the enzyme, it is induced

that the N-methyl groups in its molecule are able to combine with the active center of the oxidase. The toxicity of methylene blue, and probably that of toluylene blue too, to the oxidase, which have long been known since Philot (5) had noticed, is interpreted to be but a case of the N-methyl effect due to the existence of N-methyl groups in its molecule. Regretfully we have as yet no datum available to guess the mechanism of the effect.*

RESUMO

1. Kafeino povas kombini reverseble kun amino-oksido: la disocio-konstanto de la sterila kombinaĵo estas taksita je $10^{-2.7}$ M/l. ĉe pH 7.6 kaj 30°.

2. Kafeino tiel povas malgrandigi malhelpan agon de tiramino sur cerba spirado; la situacio tute similas al tio de benzedrino, kvankam kafeino estas ĉirkaŭ dekkvinone malpli efika ol benzedrino.

3. Ĝenerala konkludiga pruvo estas trovita, kiu indikas rilaton inter la protekta ago de kafeino sur cerba spirado kontraŭ tiramino-malhelpado kaj la centran nervon stimulantajn agojn de tiu ĉi medikamento.

4. Estas induktita, ke la afino de kafeino al amino-oksido estas atribuita je la afino de N-metilo grupoj troviĝantaj en ĝia molekulo al la enzimo.

5. Konata tokseco de metileno al amino-oksido estas klarigita per "N-metilo efiko" pro la enhavo de N-metiloj en la molekulode ĉi tiu pigmento. La disocio-konstanto de la enzimo-pigmento kombinaĵo estas je $10^{-6.5}$ M/l.

* En Passant, the activity of amine oxidase is not depressed by carbonyl reagents, viz., KCN, NH_2OH , semicarbazide, and bisulphite. Phenylhydrazine however, inhibits the enzyme, but in a competitive manner. Details will be dealt with elsewhere.

Grateful thanks are expressed to Prof. F. Egami, Nagoya University, and to Prof. H. Tamiya, Tokio University, for their stimulating encouragements and instructive advices in the course of the study.

BIBLIOGRAPHY

- (1) P. J. G. Mann and J. H. Quastel., *Biochem. J.*, **34**, 414 (1940).
- (2) Y. Oota., *Bull. Chem. Soc. Japan.*, **21**, 75 (1948); *J. Chem. Soc., Japan*, **67**, 45 (1946).
- (3) D. Richter., *Biochem. J.*, **31**, 2022 (1937).
- (4) M. A. F. Sherif., *J. Pharmacol. & Exp. Therap.*, **38**, 11 (1930).
- (5) F. J. Philpot., *Biochem. J.*, **31**, 856 (1937).



PURIFICATION OF β -GLUCOSIDASE OF ASPERGILLUS NIGER (I)

By

KOYATA NIWA

*(From the Botanical Institute, Tokyo
Bunrika University)*

(Received for publication on May 16, 1950)

A number of problems concerning the specificity of β -glucosidase have been actively discussed (1, 2, 3, 4, 5, 6), and yet most of them appear to remain unsettled. For the ultimate solution of the problem, it is desirable to isolate the enzyme in its pure state.

Helferich and his associates (1, 2) have carried out extensive investigations on sweet almond emulsin, but they have not yet been successful in isolating β -glucosidase free from other enzymes. On the basis of the finding that the activity ratios of various enzyme preparations with different purity, of sweet almond emulsin for β -d-glucoside, β -d-galactoside, β -d-xyloside and α -l-arabinoside show nearly constant value, Helferich considered that these glycosides are hydrolyzed by one and the same enzyme, β -glucosidase. Contrary to this, Miwa (7), Horikoshi (8) and Veibel (9) are inclined to the opinion that the hydrolysis of these glycosides by almond emulsin is due to specifically different enzymes corresponding to each substrate respectively.

On the other hand evidences have been given to indicate that β -glucosidase of mould is distinctly different from β -galactosidase and β -xylosidase (10, 11, 12). Besides this, some moulds produce, when cultivated under appropriate conditions, as much β -glucosidase as almond or apricot emulsin, so they can be used as suitable starting material for the purification of β -glucosidase.

In the present work, *Aspergillus niger* was used as a source of the enzyme, from which very active preparations of β -glucosidase could be obtained. They are about 60 times more active than the purest preparation of almond emulsin so far obtained by Helferich (1), when the activity is measured with phenol- β -d-glucoside as substrate. Yet they contained other enzymes than β -glucosidase in considerable amount.

1. *Method of Purification*

Tannic acid precipitation method which had been applied by several workers (13, 14, 15) to the purification of various enzymes was used with success. The purification procedure consisted generally in drying and pulverizing the mould,* extracting the enzyme from the dried powder with 10–15 times its weight of water, precipitating the enzyme with tannic acid, and washing the precipitate with acetone to remove tannic acid.

The first crude preparation was obtained by precipitation at a tannin concentration of 0.5–0.6 per cent. The enzyme extracts contain so much polyphenol-oxidase that a part of added tannin is rapidly oxidized into dark red products, forming clots together with the enzyme. These oxidation products cannot be removed by treatment with acetone and they constitute more than half an amount of the crude enzyme preparation. However, due to their insolubility in water, the colored products can be easily freed from enzyme by

* Culture of *Aspergillus niger*

Nutrient employed—Modified Hennieberg's mixture having the following composition: Cane sugar 85 g., Peptone 12.5 g., NaH_2PO_4 2 g., KNO_3 2 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g., CaCl_2 0.1 g., in 1000 ml. of distilled water.

Inocula—One ml. of aqueous suspension of spores from malt-peptone-agar slant for 500 ml. of nutrient.

Temperature and duration—30°, 3–5 days.

extracting the crude preparation with water.

Calculated on the basis of β -glucosidase content, the yield amounts to 70–80 per cent of that contained in the dried mould and to 95–100 per cent in the water extract.

100 g of air dried powder of mould with a moisture content of about 10 per cent yielded 0.2–0.4 g. of such crude enzyme preparation.

By means of this procedure alone, the activity of β -glucosidase could be elevated about 30 times of the original water extract and 200–300 times of dried powder of the mould. It is designated as tannin preparation 1.

β -Glucosidase activity of dry powder, water extract and aqueous solution of tannin preparation 1 is compared in Table I.

TABLE I

Activity of Dry Powder, Water Extract and Tannin Preparation

Enzyme preparation	f †	Ratio of f	Yield of enzyme
Dry powder	0.1	1.0	per cent 100
Water extract	1–2	10–20	70–80
Tannin preparation 1	30–40	300–400	68–80

† Specific activity f was calculated from $f = k/g \cdot \log 2$, where k is a velocity constant of the first order reaction and g is gram of enzyme in 8 ml of the reaction mixture composed of buffer ($M/5$ glycoll-HCl, pH 2, 75), substrate ($0.048 M$) and enzyme solution at the ratio of 1:1:2. In all cases of this report phenol- β -glucoside is used as standard substrate for determining β -glucosidase activity.

For further purification of the tannin preparation 1, precipitation with tannin was repeated reducing the tannin concentration gradually. Results are given in the following table.

TABLE II

Activity of Preparations in Various Stages of Purification

Repetition of precipitation	Concentration of tannin	f	Ratio of f	Yield of enzyme
	per cent			per cent
I	0.60	34.3	1.0	100.0
II	0.67	112.0	3.3	98.0
III	0.32	179.0	5.2	87.8
IV	0.25	226.9	6.6	74.8
I	0.60	31.8	1.0	100.0
II	0.36	88.4	2.8	90.2
III	0.15	224.1	7.0	83.3
IV	0.19	322.8	9.8	72.6
V	0.19	399.6	12.6	55.5

The specific activity of the best preparation which was obtained by repeating the precipitation 8 times, amounted to 6568 which was about 8000 times as active as dry powder of the mould, and the yield of β -glucosidase was no less than 35 per cent of that contained in the tannin preparation 1.

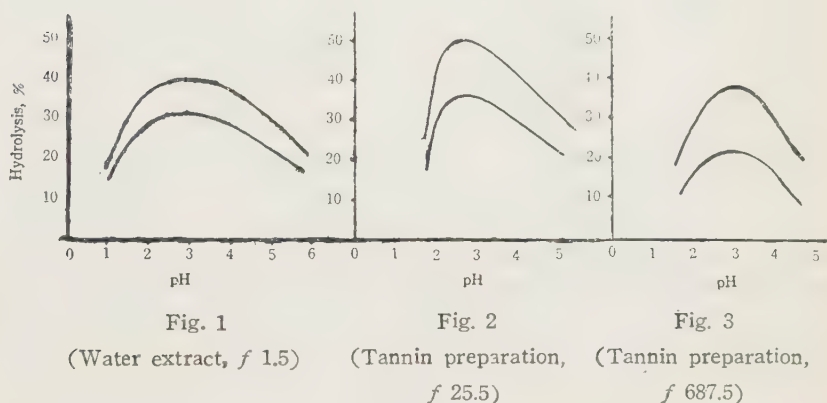
2. Constancy of Activity Ratio and pH Relationship

It has been in several instances noted that one and the same species or organ contains several "isodynamic" enzymes, which, in spite of catalyzing the same reaction, differ one another in pH optimum, relative specificity, behavior toward activators or inhibitors, etc. So, for example, in the hepatopancreas of mudsnail (*Viviparus maleatus* Reeve) there exist two kinds of β -glucosidase differing distinctly in pH optimum, relative specificity and behavior toward inhibitors (16).

If this be the case also with the β -glucosidase of *Aspergillus niger*, shift in pH optimum and activity ratio toward several substrate might happen to occur in the course of purification.

For the purpose of testing the individuality of the β -glucosidase of *Aspergillus niger*, determination of pH optimum and activity ratio toward six β -glucosides was carried out at various steps of purification.

pH Relationship of three different enzyme preparations of different purity was examined with phenol- β -d-glucoside as substrate. The results are shown in following figures.



It can be clearly seen from the figures that each enzyme preparation represents the same pH relationship with the optimum activity at about pH 3.0.*

Relative specificity of some preparations of different purity was compared with six β -d-glucosides of different aglucons and the results obtained are summarized in Table III.

* The same value of pH optimum was obtained also in the hydrolysis of salicin and o-cresol- β -d-glucoside. Interesting to note is that this value is considerably shifted to acid side as compared with the β -glucosidase of almond emulsin and Taka-diaxase.

TABLE III
Constancy of Activity Ratio (Relative Specificity) of Seven Preparations

Aglucon	Phenol	Saligenin	<i>o</i> -Cresol	<i>p</i> -Cresol	<i>p</i> -Nitro-phenol	Vanillin
Enzyme prep.						
1. (f. 41.5)	1.00†	0.36	0.034	1.10	0.48	0.48
2. (f. 88.4)	1.00	0.35	0.034	1.15	0.44	0.43
3. (f. 224.2)	1.00	0.35	0.034	1.11	0.38	0.38
4. (f. 315.0)	1.00	0.33	0.033	1.13	0.45	0.44
5. (f. 510.0)	1.00	0.35	0.030	1.21	0.42	0.40
6. (f. 690.0)	1.00	0.39	0.034	1.18	0.48	0.46
7. (f. 821.0)	1.00	0.38	0.034	1.18	0.46	0.41

† The rate of hydrolysis of the phenol- β -d-glucoside is taken as unity.

These results indicate that different preparations show quite constant relative specificity.

Partially purified enzyme preparations by means of acetone precipitation and salting out also showed no change of relative specificity (Table IV).

TABLE IV
Activity Ratio of some other Enzyme Preparations

Enzyme pre-paration	Phenol	Saligenin	<i>o</i> -Cresol	<i>p</i> -Cresol	<i>p</i> -Nitro-phenol	Vanillin
A	1.00	0.36	0.034	1.10	0.48	0.46
B	1.00	0.41	0.036	1.05	0.49	0.50
C	1.00	0.42	0.030	1.15	0.58	0.46
D	1.00	0.37	0.037	1.14	0.49	0.43
E	1.00	0.37	0.037	1.07	0.47	0.38

A. Tannin preparation, f. 41.5; B. Prepn. A precipitated with acetone at 77.8 percent, f. 25.4; C. Prepn. A. precipitated with acetone at 66.7 per cent, f. 58.8; D. Prepn. A salted out with ammonium sulfate at 0.6 saturation, f. 2.1; E. Supernatant solution of D salted out with ammonium sulfate at 0.75 saturation, f. 9.7.

3. *Behavior of Other Carbohydases in Purification Process*

In addition to β -glucosidase, other carbohydases are present in the crude enzyme preparation. Behavior of these enzymes in the course of purifications of β -glucosidase was traced and following results were obtained (Table V).

TABLE V

Ratio of Activity for 6 Carbohydases in Several Preparations of Different Purity

Enzyme prep.	Water Extract	Tannin preparation					
		I	II	III	IV	V	VI
Enzymes f	10	31.8	88.4	224.1	315.0	939.0	821.0
β -Glucosidase†	1.000††	1.000	1.000	1.000	1.000	1.000	1.000
α -Glucosidase†	0.034	0.011	0.009	0.003	0.003	0.002	0.001
Maltase	0.240	0.180	0.180	0.056	0.051	0.037	0.011
β -Galactosidase†	0.014	0.012	0.014	0.013	0.009	0.009	0.004
α -Galactosidase†	0.376	0.190	0.130	0.085	0.054	0.058	0.019
Saccharase	3.680	2.500	2.170	1.340	0.910	0.500	0.212

† Phenol-glycosides are used as substrate.

†† Activity of β -glucosidase is taken as unity.

As shown in the above table, carbohydases other than β -glucosidase contained in the initial crude preparation diminished more or less as the purification proceeded. Accordingly this method appears to represent an excellent one not only to elevate the purity of β -glucosidase, but also to remove accompanying carbohydases.

SUMMARY

1. Tannin precipitation method is an excellent one for the purification of β -glucosidase of *Aspergillus niger*. The most active preparation obtained by this means represented the activity of 8000 times that of starting material, the dry powder of the mould.

2. pH Relationship and relative specificity did not shift in the course of purification. This suggests that this mould contains only

one kind of β -glucosidase in respect of relative specificity.

3. As this purification method permits to increase the purity of β -glucosidase in preference to other accompanying carbohydrases, it may be regarded as valuable one for separating β -glucosidase from them.

The cost of this research was defrayed in part by a grant from the Ministry of Education given to T. Miwa.

REFERENCES

1. Helferich, B., *Ergeb. Enzymforsch.*, **2**, 74 (1933).
2. Helferich, B., *Ergeb. Enzymforsch.*, **7**, 83 (1938).
3. Hofmann, E., *Biochem. Z.*, **285**, 429 (1936).
4. Miwa, T., Cheng, C., Fujisaki, M. & Toishi, A., *Acta Phytochim.*, **10**, 155 (1937).
5. Miwa, T., Cheng, C., Miwa, A., Fujisaki, M. & Ouchi, K., *Sci. Rep. Tokyo Bunrika Daigaku, B.*, **6**, 41 (1942).
6. Pigman, W. W., *Adv. Enzymol.*, **4**, 41 (1946).
7. Miwa, T. *et al.*, To be published.
8. Horikoshi, K., *J. Biochem.*, **35**, 39 (1942).
9. Veibel, S. & Lillielund, H., *Z. physiol. Chem.*, **253**, 55 (1938).
10. Nishizawa, K., *Sci. Rep. Tokyo Bunrika Daigaku, B.*, **6**, 43 (1942).
11. Miwa, T., Nishizawa, K., Kinugawa, H. & Miwa, A., *Medicine and Biology*, **1**, 61 (1942) (In Japanese).
12. Miwa, T. & Miwa, A., *Medicine and Biology*, **2**, 396 (1942) (In Japanese).
13. Willstätter, R. & Pollinger, *Ann. Chem.*, **430**, 286 (1923).
14. Weidenhagen, R., *Ver. Dtsch. Zuckerind.*, **81**, 501 (1931).
15. Helferich, B., *Z. physiol. Chem.*, **205**, 91 (1932).
16. Niwa, K., Unpublished observation.

STUDIES ON THE CONJUGATED LIPIDS. 1. ON THE CONFIGURATION OF CEREBROSIDES.

By

TEISHI NAKAYAMA

*(From the Department of Medical Chemistry, Hokkaido University, School
of Medicine. Director : Prof. Morio Yasuda)*

(Received for publication on June 6, 1950)

INTRODUCTION

Of conjugated lipids, cerebroside or galactolipins may be mentioned as characteristic ones together with phospholipids. By hydrolysis cerebroside gives particular fatty acids, fatty base named sphingosine and galactose which are unique compared with other lipids.

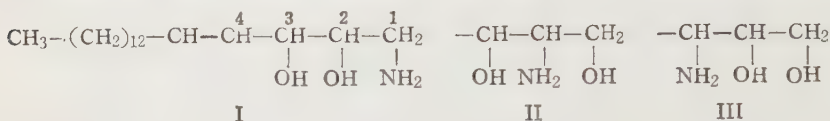
The cerebroside so far well known are cerebrin, kersin, nervon and oxynervon. Cerebrin sulphuric acid by Blix (1933) (1) and gangliosides by Klenk (1942) may be added to the above.

In connection with the study of the determination method of these lipids, the author has isolated the most commonly cerebroside—cerebrin, and tried to detect its chemical configuration.

For this purpose, it is necessary to ascertain firstly the configuration of hydrolysis products of cerebrin. There is no question about the configuration of fatty acids and galactose. Regarding sphingosine, however, its configuration was reported by Carter (2) and Ohno (1944) (3) that the two hydroxyl radicals and one amino radical are not arranged in sphingosine molecule as concluded by Klenk and Diebold (1931) (4).

Moreover, the position of the linkage between sphingosine and galactose has not been elucidated yet.

Sphingosine was firstly isolated by Thudicum and Thierfelder (1901, 1906) (5), as a component of cerebrosides and sphingomyeline, and later studied by Levene (1912-1916) (6) and Klenk (1928-1930) (5), especially by Klenk who gave following formula for sphingosine.

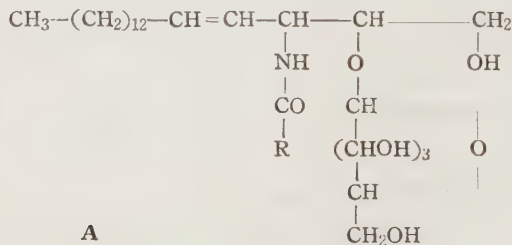


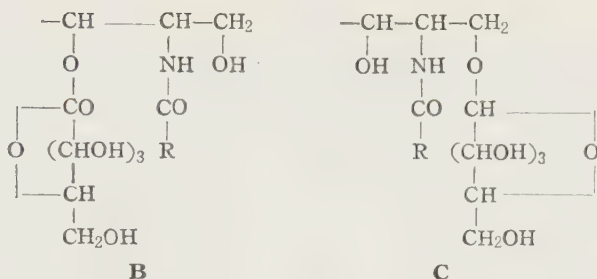
However, I) the positions of two hydroxyl groups and one amino group (*), II) stereoisomers due to two asymmetric carbon atoms at C₂ and C₃, III) cis-trans isomerism owing to the double bond in C₄=C₅ were not made clear.

(*) Klenk and Diebold hydrolysed the nitrogen containing substances obtained by ozonic decomposition of triacetylsphingosine, which were oxydized with bromine to get dioxyamino butylic acid, and this acid, after treatment with hydrogen iodide, showed melting point of 280°-285°, and they concluded that the above acid was α-amino acid so that sphingosine must have the (III) type of configuration.

Carter (1947) (2) and Ohno (1944) (3) had independently tried the oxydation of the sphingosine and their derivatives with periodic acid and lead tetraacetate respectively, and concluded that the configuration of sphingosine was not of (III) type as Klenk claimed, but it should be of (II) type. Moreover, Ohno (1948) (7) found that the double bond in C₄=C₅ is to be trans type.

The configuration of the cerebrosides after Klenk (1931) shown below formula (A) should be corrected as (B) or (C).

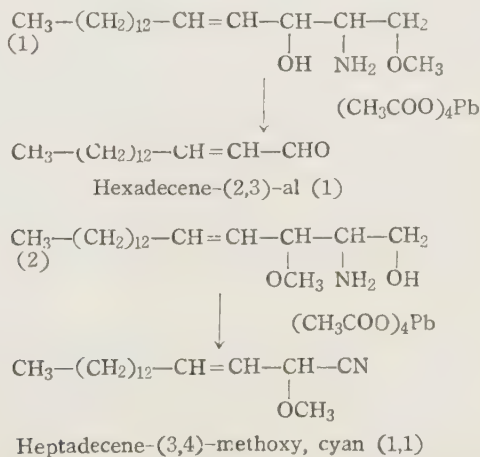




(*) Klenk described that the glucosidic linkage was unknown whether it is in C₁ or C₂.

(*) R is fatty acid radical; in case of cerebrin it is CH₃(CH₂)₂₁CHOHCO—

If free hydroxyl groups of cerebrosides be substituted with methyl radical by methylation, and then hydrolysed, we can obtain O-methylsphingosine of the formula (1) from above (B), and the formula (2) from (C). Applying the lead tetraacetate oxydation of Criegee (1931) (8) to them as Ohno (1948) (7) carried out, one could obtain α - β -unsaturated palmitic aldehyde from (1), and a nitril of seventeen carbons from the formula (2) as indicated below:



Thus, the position of glucosidic linkage in the cerebroside molecules can be known easily. The author attempted to make clear this fact by the isolation and methylation of cerebrin.

EXPERIMENTAL

Isolation of Protagon from Pig Brain.—15 kg. of fresh pig brain were finely minced and put in acetone of its three times volume overnight with occasional stirring, then filtered. This treatment was repeated several times (removal of fats and cholesterol). The residue was sufficiently extracted with dry ether using Soxlet apparatus (removal of phosphatides). The residue was evaporated to almost dryness, then extracted with chloroform and methanol mixture (1:3 vol.) using Soxlet apparatus. The extract was distilled under vacuum, added to ether and left overnight at -15° . Ether was filtered off at the same temperature, and the insoluble part which is crude protagon fraction was thus obtained weighing 380 g. For the purification it was extracted with five times volume of boiling alcohol (85%) and filtered still hot. Precipitate appeared on cooling which was separated off. The procedure was repeated until no more precipitate was obtained on cooling. Thus the author could obtain about 260 gm. of protagon as weak yellowish powder.

Isolation of Cerebron from Protagon Fraction—260 g. of protagon were dissolved in five times volume of pyridine (b.p. 115° – 116°) stirred occasionally at 60° and cooled overnight at 0° , after that the precipitate was filtered. The precipitate was next treated with equal volume of pyridine and filtered. Both of mother lipids were evaporated under vacuum. The cerebroside fraction thus obtained, were recrystallised twice from three times volume of Chloroform methanol mixture (3:1 vol.) whereby 40 g. of pale yellowish powder was obtained.

This powder was recrystallised repeatedly by dissolving in boiling chloroform-methanol mixture and subsequent cooling. The ratio of chloroform and methanol was changed so that the amount of methanol was gradually increased against chloroform. First, the powder was dissolved in three volumes of chloroform-methanol (2:1 vol.) and recrystallised by cooling. The same was repeated in five volumes of C-M (chloroform-methanol) mixture (1:1), five volumes of C-M

(1:3), and ten volumes of C-M (1:9).

In each case of different chloroform-methanol mixture, recrystallisation was repeated three or four times. Finally about 5 g. of cerebron (phrenosin) were obtained.

This substance was an amorphous snow white crystal, insoluble in water, ether and petroleum ether, soluble in alcohol a little, but in chloroform and pyridine very easily. The selenite-plate-test of Rosenheim (1914) (9) showed that this crystal was pure cerebron, kersin being not contaminated.

$$[\alpha]_D^{12} = 4.18^\circ \text{ (in pyridine C=3\%)}$$

Substance	45.085 mg.	N found	1.72% (0.686 ml.)
		calc.	1.69%

Methylation of Cerebron—Purdie method (1903) was applied. It seemed necessary to investigate whether the oxygen ring of the galactose in cerebron is 1-5 or 1-4 bond as Pryde and Humphreys (1924) (10) reported. The reaction was very difficult at the first stage of the methylation, owing to the small solubility of the cerebroside in methyl iodide, so that it was necessary to add chloroform to some extent. When the methylation proceeded, these difficulties were gone, because the reaction products became more and more soluble in organic solvents.

Eight methylation was needed to complete the methylation. Methoxyl value was slightly lower than that of the theoretical, which resembled to the result of Pryde and Humphreys, though melting point did not agree.

Thus, the author got about 2.5 g. of hexamethylcerebron from 3.5 g. of cerebron. This substance was a snow white powder and melted at 66.5°–67.5°, insoluble in water and petroleum ether, soluble in all other organic solvents. The results of methylation are indicated below;

Methylation of Cerebron

No. of methylation	Reagent	Extraneous solvent	Extractive solvent	Methoxyl (%)	m. p.
1	27 ml. CH ₃ I	35 ml. CHCl ₃	hot	—	50°–60°
	51 g. Hg ₂ O		alcohol		
2	30 ml. CH ₃ I	10 ml. CHCl ₃	"	—	40°–45°
	27 g. Ag ₂ O				
3	30 ml. CH ₃ I	—	"	—	—
	30 g. Ag ₂ O				
4	30 ml. CH ₃ I	—	"	16.2	56°–59.5°
	30 g. Ag ₂ O				
5	20 ml. CH ₃ I	—	"	—	59°–63°
	20 g. Ag ₂ O				
6	20 ml. CH ₃ I	—	alcohol	17.8	63°–65°
	20 g. Ag ₂ O		and ether		
7	20 ml. CH ₃ I	—	"	—	63°–65°
	17 g. Ag ₂ O				
8	20 ml. CH ₃ I	—	"	18.8	66.5°–67.5°
	27 g. Ag ₂ O				

(Hexamethylcerebron requires 20.41% of methyl.)

Hydrolysis of Hexamethylcerebron—1.103 g. of hexamethylcerebron were dissolved in 55 ml. of 10% (vol.) sulphuric acid containing methanol, boiled for seven hours on the water-bath, and cooled overnight at 0°. Methyl esters of fatty acids have precipitated in large amount which were separated by filtration and washed with small amount of ice cold methanol (fatty acid fraction).

The filtrate was added to about twice as much water, and heated on the water-bath to get rid of methanol by evaporation. After cooling, the residue was treated several times with petroleum ether to remove the traces of fatty acids, then neutralized with dilute sodium hydroxide, and left overnight at 0°.

The resulting precipitate which is sphingosine fraction was separated by centrifuging (2500 r.p.m.) for one hour at low temperature; the supernatant liquid was decanted from which sugar fraction was obtained.

Fatty Acid Fraction—This fraction was dissolved in petroleum ether, and centrifuged (the insoluble part is sphingosine). The supernatant was distilled under vacuum. The residue was added to water and methanolic KOH solution, and heated on the water-bath for an hour. Fatty acid was saponified by this procedure while methanol was evaporated off. The remaining watery solution of soap was transferred to a separating funnel, acidified with 2 N H₂SO₄ and the fatty acid freed thereby was extracted with ether. The ether layer was freed from water with Na₂SO₄ after that it was evaporated under vacuum. Crude fatty acid fraction was thus obtained weighed 0.44 g. A part of this fatty acid was recrystallised from benzene. A white oily solid was obtained which melted at 65°-67.5°, and it was identified as α -methoxy-lignoceric acid as shown below.

Substance	3.108 mg.	OCH ₃	found 7.52% (N/50 Na ₂ S ₂ O ₃ 2.26 ml.)
α -methoxy-lignoceric acid			calcd. 7.79%

A small part of this recrystallised fatty acid was dissolved in ammonia containing methanol, and evaporated to dryness on the water-bath. The ammonium salt of fatty acid was redissolved in methanol and added to calculated amount of methanol solution of silver nitrate. White cloudy precipitate of silver salt of fatty acid was produced which was separated by filtration and dried. Silver content was determined by ash determination method,

Substance	8.203 mg.	Ag found 1.823 mg.
silver salt of α -methoxy-lignoceric acid		calcd. 1.751 mg.

Sphingosine Fraction—This fraction weighing 0.27 g. was dissolved in 1N alcoholic sodium hydroxide, alcohol was evaporated and the residue was suspended in small amount of water in separating funnel, then added to ether and shaken. The ether layer was separated, dried with anhydrous Na₂SO₄ and evaporated under vacuum. The residue was dissolved in small amount of absolute alcohol, to which was added an absolute alcoholic sulphuric acid drop by drop until

the solution had lost their basic reaction against litmus paper. The neutralised solution was cooled for an hour at 0° , and the resulting crystall of o-methyl-sphingosine sulfate was filtered and washed with small amount of ice cold absolute alcohol. The above procedure was repeated to get about 0.2 g. of o-methyl-sphingosine as oval yellowish powder.

Substance	2.915 mg.	OCH_3	found	7.60%	(N/50 $\text{Na}_2\text{S}_2\text{O}_3$ 2.14 ml.)
o-methyl-sphingosine sulfate			calcd.	7.54%	

Sugar Fraction—The sugar fraction obtained by the procedure noted elsewhere on this paper was taken into an extraction apparatus and extracted with ether. After seventy hours of extraction the ether layer was separated, dried over anhydrous Na_2SO_4 and evaporated under vaccum. The resulting oily substance was boiled with 10 ml. of 10% methanol solution of HCl on the water-bath for two hours, then the solution was distilled under vaccum to remove methanol and HCl. The residue was transfered into a small distillation apparatus and distilled under vaccum. About 0.1 g. of colourless perfuming oily substance which boiled at 170° – 180° /13–12 mm. Hg was obtained.

This oily substance had a refractive index of $n_D^{60}=1.4528$ which agreed with that of 2, 3, 4, 6-tetramethyl-methyl-galactoside, b.p. 176° /13 mm. Hg, $n_D^{120}=1.4484$.

Oxydation of O-Methyl-Sphingosine with Lead Tetraacetate

0.20 g. of O-methyl-sphingosine sulfate described above was dissolved in 10 ml. of anhydrous benzene. To this solution was added 0.35 g. (1.5 mol.) of lead tetraacetate after that the solution was warmed at 50° for three hours under reflux. After cooling, small amount of ethylene glycol was added to decompose excess lead tetraacetate. Then the benzene was washed thoroughly with water to remove lead salt. The benzene layer was transfered to another

flask and dried over anhydrous Na_2SO_4 . The residue after evaporating the benzene under vacuum did not show positive reaction by fuchsin-sulphurous acid solution (Schiff's reaction). The author assumed that the product was a nitril. It was therefore treated with 5 cc. of alcohol containing 0.3 g. of potassium hydroxide and boiled under reflux on the water-bath for hydrolysis. When the boiling was continued for two or three hours, the author found much evolution of ammonia gas, and it was further identified by Nessler's reagent and ammonium chloride test. The boiling was continued until no more ammonia gas evolved from the top of the condenser (about 15 hrs.), then after cooling, added to alcoholic sulphuric acid solution drop by drop, till the solution showed slightly acid to litmus paper. To this solution added 40 ml. of water and was set on the water-bath to evaporate alcohol. To the fatty acid suspending solution thus obtained was added ether in separating funnel. After sufficient shaking the ether layer was separated, dried over anhydrous Na_2SO_4 and evaporated under vacuum. The oily residue thus obtained weighed about 0.1 g.. As the isolation of free fatty acid was difficult, owing to its slight volume, the residue was treated at once with ammonia containing methanol and the residue, after evaporation of excess methanol and ammonia, was redissolved in methanol. To this added methanol solution of silver nitrate. White, cloudy precipitate of silver salt of fatty acid was filtered and dried (yield about 30 mg.).

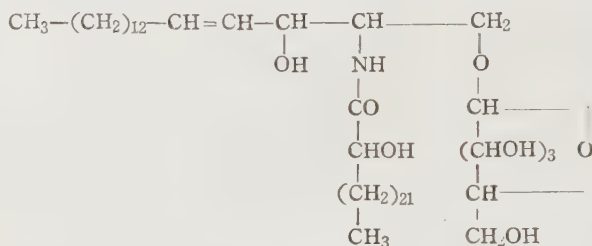
Substance	3.41 mg.	OCH_3	found 7.82% (N/50 $\text{Na}_2\text{S}_2\text{O}_3$ 2.6 ml.)
			calcd. 7.65%
	13.370 mg.	Ag	found 1.840 mg.
Ag salt of α -methoxy-(β - γ)-heptadecenic acid : calcd. 1.963 mg.			

The investigation of the physical properties and chemical analysis of the free acid could not be tried owing to the shortage of the material.

SUMMARY

(1) The author has taken up cerebrin as a most representative cerebroside which was isolated from pig brain. By making use of Purdie method of methylation, hexamethylcerebrin was obtained, and the hydrolysis products of it were studied regarding fatty acid, sphingosine and sugar fractions respectively.

The author reached the conclusion that cerebroside, as far as cerebrin is concerned, has a glucosidic linkage between galactose and sphingosine at the C_1 position of sphingosine and the structure of cerebrin must be as follows:



Note. Stereoisomers due to the asymmetric carbon atom of C_2 and C_3 were not yet studied.

(2) By oxydation of O-methyl-sphingosine with lead tetraacetate was demonstrated that the positions of two hydroxyl groups and one amino group in sphingosine molecule are not as Klenk and Diebold claimed, but they are, as Carter and Ohno concluded, that two hydroxyl groups are attached to C_1 and C_3 being the amino group in C_2 position of sphingosine.

The author wishes to express his deep indebtedness to Prof. M. Yasuda for helpful criticism of this work.

REFERENCES

- (1) Blix, G.: *Z. physiol. Chem.*, **219**, 82 (1933).
- (2) Carter, H.E.: *J. Biol. Chem.*, **170**, 285 (1947).
- (3) Ohno, K.: *J. Japanese Biochem. Soc.* **18**, 151 (1944) (Japanese).

- (4) Klenk, E. & Diebold, W.: Z. physiol. Chem., **198**, 25 (1931).
- (5) Thierfelder, H. & Klenk, E., Die Chemie der Cerebrosid und Phosphatid (1930).
- (6) Levene, E. A. & West, J.: J. Biol. Chem., **24**, 63 (1916).
- (7) Ohno, K.: J. Jap. Biochem. Soc. **20**, 38 (1944) (Japanese).
- (8) Criegee, Z.: angew. Chem., **50**, 153 (1937).
- (9) Rosenheim, O.: Biochem. J., **8**, 110 (1914).
- (10) Pryde, J. & Humphreys, R. W.: Biochem. J., **18**, 661 (1924).



THE FATE OF BRANCHED CHAIN FATTY ACIDS IN ANIMAL BODY. I. A CONTRIBUTION TO THE PROBLEM OF "HILDEBRANDT ACID".*

By

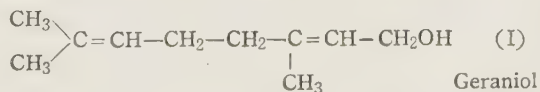
MITIZO ASANO and TAMIO YAMAKAWA

(From the 7th Department of the Institute for Infectious Diseases, University of Tokyo.)

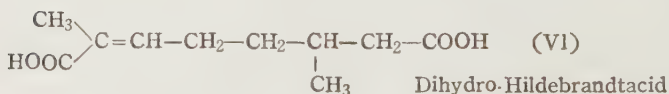
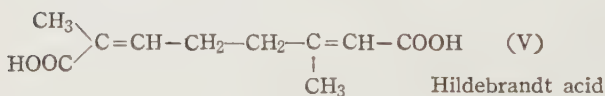
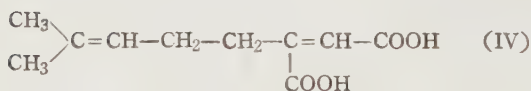
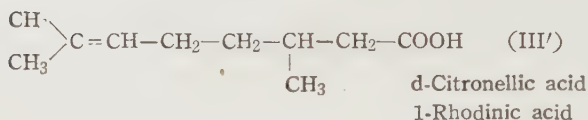
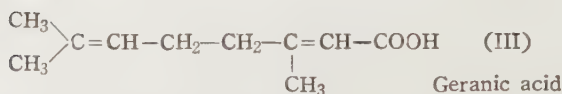
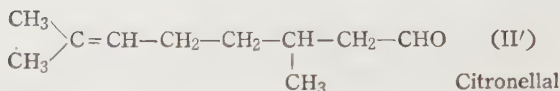
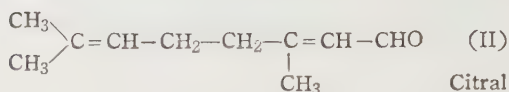
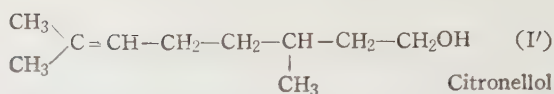
(Received for publication June 6, 1950.)

In 1901, Hildebrandt (1), while studying pharmacological action of aliphatic terpenes, isolated an acid, m.p. 187°, from the urine of a rabbit administered citral. Since this was proved to be a dicarboxylic acid, he proposed for it the structural formula (IV). Furthermore, he found another acid, m.p. 96°, from its mother liquor.

Later, in 1933, another opinion about this problem was raised by R. Kuhn (2). He applied Verkade's ω -oxidation theory (3), introduced in the preceding year, to this problem, gave the higher melting acid, the formula (V) instead of (IV), and confirmed his assumption by chemical degradation and synthesis (4). This was named "Hildebrandt acid". An acid with lower melting point was also isolated by Kuhn and, in addition, found to be optically active giving $[\alpha]_D = +15^\circ$. Hence, he assumed that citral suffered an α - β -saturation by asymmetric hydrogenation together with ω -oxidation in animal body. Accordingly, he gave it formula (VI) and confirmed by experimental evidences. This was "dihydro-Hildebrandt acid".



* This was previously published in Japanese in J. Pharm. Soc., Japan, 68, 147 (1948).



In this case, dihydro-Hildebrandt acid excreted became α - β -saturated in animal body, Kuhn assumed that if α - β -saturated aliphatic terpenes, such as (I'), (II') or (III'), were administered, the yield of dihydro-Hildebrandt acid would be enhanced, and he gave citronellal (II') to a rabbit *per os* (5). However, he failed to isolate the expected substance, and instead obtained a different substance which he considered to have been formed from citronellal by the action of acidity of the gastric juice.

Following the works of Kuhn, under the direction of similar idea, F. G. Fischer, who had been studying at that time on phytochemical reduction by fermenting yeast, gave citronellol (I') to a rabbit and obtained an acid from its urine, which, contrary to his expectations, did not melt at ca. 100° , but fused at $84-5^{\circ}$. The optical activity of this acid was $[\alpha]_D = +8^{\circ}$. As a result of chemical analysis, he concluded that this substance must have the same structural formula as dihydro-Hildebrandt acid.

The authors hitherto obtained an apparently unsaturated dicarboxylic acid after the administration of a saturated branched-chain fatty acid to rabbits. Later, we took up the determination of the position of the double bond. We assumed that if α - β -saturated aliphatic terpenes such as (I'), (II') or (III') were administered and suffered α - β -desaturation simultaneously with ω -oxidation, Hildebrandt acid would be obtained. By this experiment, we attempted to demonstrate biochemical desaturation of fatty acids.

Fortunately, 1-rhodinic acid was supplied by the courtesy of the Takasago Perfumery, Co. We injected this acid to a rabbit subcutaneously, in the form of an aqueous solution of its sodium salt. The collected urine was concentrated under reduced pressure, acidified and extracted continuously with ether. By the vacuum distillation of the acid portion of the ethereal extract, we obtained, at first, benzoic acid, and then a viscous, slightly yellowish liquid. This product solidified soon and after repeated recrystallisation from water, showed m. p. $101-2^{\circ}$, $[\alpha]_D^{10} = -13.5^{\circ}$. We named it "R-diacid". The melting point of this substance was approximately the same as that of dihydro-Hildebrandt acid previously obtained by the German authors. While that obtained by the latter was dextrorotatory, our product was levorotatory.

Then, we administered d-citronellic acid, prepared by the oxidation of d-citronellal, to a rabbit and obtained an acid, m. p. $102-4^{\circ}$, $[\alpha]_D = +15.6^{\circ}$. ("C-diacid").

As already mentioned, Kuhn failed to obtain dihydro-Hildebrandt acid after oral administration of d-citronellal, but we succeeded in isolating a substance which proved to be identical with "C-diacid" by the subcutaneous injection of d-citronellal.

At first, R-diacid and C-diacid appeared to be the same on account of their melting points and the values of analysis, but the mixed melting point determination showed the two substances to be different. Therefore, it seemed probable that one of these two substances was not dihydro-Hildebrandt acid.

In order to explain this complicated state, we administered geraniol to rabbits and isolated from the urine, dihydro-Hildebrandt acid, m.p. 100-2°, $[\alpha]_D = +14.1^\circ$, besides Hildebrandt acid, m.p. 190-1°.

By the mixed melting point determination of the three substances with each other, it was confirmed that C-diacid was identical with the *true* dihydro-Hildebrandt acid. R-diacid, therefore, was thought to be the optical antipode of *true* dihydro-Hildebrandt acid, which would explain the fact that the mixed melting point showed depression because of formation of a racemate or a conglomerate.

We take pleasure in acknowledging our indebtedness to the Takasago Perfumery, Co., for supplying all the substances used for this study.

We are also indebted to Miss. S. Suzuki and Miss. S. Irie for undertaking microanalyses.

EXPERIMENTAL.

In general, we followed the procedure described by F. G. Fischer (6).

Acid samples were neutralized exactly with sodium hydroxide, corrected to pH 7.2-7.4 by the addition of phosphate buffer solution, and 0.5 g. of them were injected subcutaneously twice a day into normal rabbits, weighing 2-3 kg.

Neutral samples, such as aldehyde or alcohol were injected un-

treated. After a total of ca. 10 g. had been administered, the urine was collected during the next 48 hours, acidified with hydrochloric acid to congo reaction and concentrated under reduced pressure. 200 ml. of the concentrated dark brown urine was extracted continuously with ether for ca. 40 hours. Ethereale xtract was separated into acid and neutral portions by shaking with 5 percent sodium carbonate solution. By the vaccum distillation of the acid portion, solid acid was isolated at first. This melted at 120° and was readily identified as benzoic acid. Further distillation gave viscous yellowish liquid at $170-180^{\circ}$ under a pressure of 0.1 mm. Hg, which solidified soon. This was purified by recrystallisation from water or ligroin.

1-Rhodinic acid, a constituent of *Chamaecyparis taiwanensis* (7), was rectified, and the portion, boiling out at $139-140^{\circ}$ (9 mm.) and giving $\alpha_D^{15} = -6.44^{\circ}$ (1 dm.), $n_D^{25} = 1.4578$, was used. From the urine collected from a rabbit injected with 8.5 g. of 1-rhodinic acid was isolated 3.45 g. of yellowish solid, b. p._{0.2} $174-186^{\circ}$, which gave m. p. $102-103.5^{\circ}$ when recrystallized from water to colorless powder, readily soluble in ether, alcohol, ethyl acetate and acetone, and slightly soluble in petroleum ether, ligroin and water.

Titration: 4.450 mg. of the substance dissolved in neutral alcohol, with phenolphthalein as the indicator, required 4.65 ml. of 0.01 *N* NaOH.

Calcd. for $C_{10}H_{16}O_4$: COOH 45.00%. Found: 45.05%.

Rotation: 300.09 mg. of the substance was dissolved in 2 *N* NaOH and made up to 10 ml. gave, in a 1 dm. tube, a reading of -0.406° , hence $[\alpha]_D = -13.5^{\circ}$.

d-Citronellic acid, b. p.₁₁ $141-3^{\circ}$, $\alpha_D = +6.31^{\circ}$ (1 dm.), was administered to a total of 10 g. to a rabbit. 6.07 g. of viscous yellowish oil, b. p._{1.2} $165-190^{\circ}$, was isolated from its urine. The oil solidified soon, and was recrystallized from water to colorless powder, m. p. $103-4^{\circ}$.

Titration: 3.980 mg. of the substance dissolved in neutral alcohol, with phenolphthalein as the indicator, required 3.98 ml. of 0.01 *N*

NaOH.

Calcd. for $C_{10}H_{16}O_4$: COOH 45.00%. Found: 45.03%.

Rotation: 300.22 mg. of the substance was dissolved in 2*N* NaOH and made up to 10 ml. gave, in a 1 dm. tube, a reading of $+0.47^\circ$, hence $[\alpha]_D = +15.6^\circ$.

d-Citronellal, b.p.₁₇ 97° , $n_D^{13} = 1.4518$, $\alpha_D = +3.64^\circ$, was injected into a rabbit to the total of 11 g. From the urine, 2.33 g. of an acid, b.p._{0.05} $160-180^\circ$, was obtained, which melted at $103-4^\circ$, when recrystallized from water.

Rotation: 200.0 mg. of the substance was dissolved in 2 *N* NaOH and made up to 10 ml. gave, in a 1 dm. tube, a reading of $+0.306^\circ$, hence $[\alpha]_D = +15.3^\circ$.

Geraniol, b.p.₁₇ $120-2^\circ$, $n_D^{13} = 1.4755$, $\alpha_D = -2.89^\circ$ (somewhat impure) was given during 13 days to a total of 22 g. 5.3 g. of viscous liquid was obtained from the urine at $170-195^\circ$ (1.5 mm.), which solidified the next morning. A portion, which was insoluble in water, was recrystallized from alcohol to colorless needles, melting at $190-1^\circ$ (the so-called Hildebrandt acid). From its mother liquor, an acid more readily soluble in water, dihydro-Hildebrandt acid, m.p. $102-4^\circ$, was obtained.

Titration: 3.990 mg. of the substance dissolved in neutral alcohol, with phenolphthalein as the indicator, required 3.98 ml. of 0.01 *N* NaOH.

Calcd. for $C_{10}H_{16}O_4$: COOH 45.00%. Found: 44.89%.

Rotation: 300.010 mg. of the substance was dissolved in 2 *N* NaOH and made up to 10 ml. gave, in a 1 dm. tube, a reading of $+0.425^\circ$, hence $[\alpha]_D = +14.1^\circ$.

REFERENCES

- (1) Hildebrandt, H., Arch. exp. Path. & Pharmacol., **45**, 110 (1901).
- (2) Kuhn, R. & Livada, K., Z. physiol. Chem., **220**, 235 (1933).
- (3) Verkade, P.E. & Lee, J. van der., Z. physiol. Chem., **215**, 225 (1933).

- (4) Kuhn, R., Köhler, Fr. & Köhler, L., Z. physiol. Chem., 242, 171 (1936).
- (5) Kuhn, R. & Köw, I., Z. physiol. Chem., 254, 139 (1938).
- (6) Fischer, F.G. & Bielig, H.-J., Z. physiol. Chem., 266, 73 (1940).
- (7) Kafuku, K., Nozoe, T. & Hata, Bull. Chem. Soc. Japan., 6, 40, 111 (1931).



THE FATE OF BRANCHED CHAIN FATTY ACIDS IN ANIMAL BODY. II. CHEMICAL CHANGES OF SOME SYNTHETIC BRANCHED- CHAIN FATTY ACIDS IN VIVO.*

By

MITIZO ASANO and TAMIO YAMAKAWA

(From the 7th Department of the Institute for Infectious
Diseases, University of Tokyo).

(Received for publication June 6, 1950).

In the previous report (1), we added some new facts to the problem of Hildebrandt acid. However, that work was, as it were, a matter of secondary importance, as we were engaged in the investigation of chemical changes of branched-chain fatty acids *in vivo*.

As is well known, branched-chain fatty acids form a group of biologically very interesting substances; e. g., they show considerably effective antibacterial activity against *Staphylococcus aureus* (2) and certain acid-fast bacilli (3) *in vitro*, and in nature, they occur as the constituent of some bacteria (4-5), lanolin (6) and of cat kidney (7). Recently, they have been investigated from the standpoint of their effects on atypical growth and proliferation of cells, as in phthioic acid in tubercle formation (8), and phytomonic acid in the crown-gall disease in plant (9). Since the constituents of bacteria, which attacks an organism and dies therein, are considered to be catabolized in accordance with biochemical laws ruling in the host organism, study of the metabolism of branched-chain fatty acids in animal body may be a matter of bacteriological interest.

Already in 1924, Kay and Raper (10) noted that ω -phenyl fatty

* This was previously published in J. Pharm. Soc., Japan. 63, 138 (1949) in Japanese.

acid, which possessed a methyl group at α -position to carboxyl as a side chain, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}-\text{COOH}$, was changed *in vivo* to benzoic acid,



$\text{C}_6\text{H}_5-\text{COOH}$, showing that the breakdown of the substance took place irrespective of the presence of a side chain. In case where the

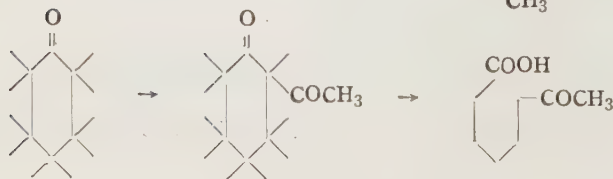
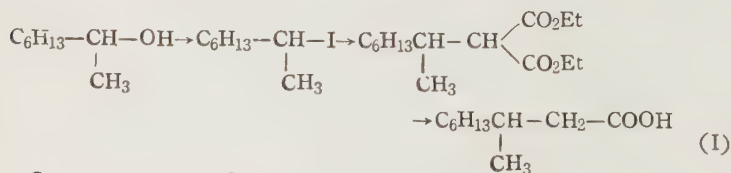
methyl group lies at β -position, Kuhn and Livada (11) found that $\text{C}_6\text{H}_5-\text{CH}-\text{CH}_2-\text{COOH}$ was excreted unchanged in the urine, in-

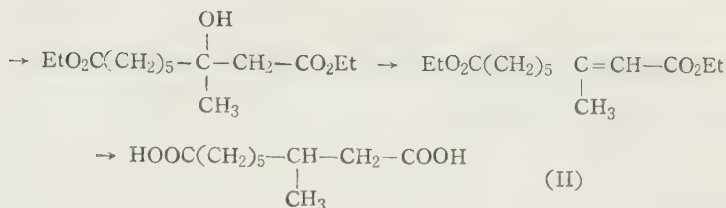


dicating that the side chain at β -position inhibited the breakdown.

In the present communication, we report some instances of the breakdown of branched chain fatty acids, in which the length and position of the side chain are different.

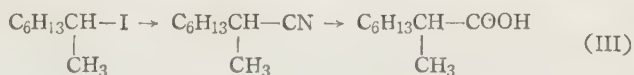
(1) Methyl group at β -position. We prepared 3-methylpelargonic acid (I) and injected it subcutaneously into a rabbit in the form of an aqueous solution of its sodium salt. After administration of 12 g. of the substance, the collected urine was treated in a manner similar to that described in the previous report (1). At first, benzoic acid was isolated, but later 3.8 g. of viscous yellowish liquid was obtained. The mass was methylated with diazomethane, rectified and then saponified. This purified acid gave *p*-bromophenacyl ester of m. p. 100–101.5°. Upon elemental analysis, it was assumed to be bis-*p*-bromophenacyl ester of C_{10} -dicarboxylic acid. For the purpose of identification, 3-methylazelaic acid (II) was synthesized as follows:



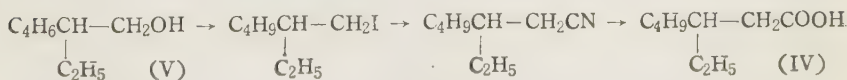


The bis-*p*-bromophenacyl ester of this synthetic dicarboxylic acid melted at 101–2°, and the mixed melting point with the derivative of the acid from urine showed no depression, by which the so-called ω -oxidation was verified.

(2) Methyl group at α -position. 2-Methylcaprylic acid (III) was prepared and a total of 10 g. was injected into a rabbit. From its urine 1.08 g. of viscous acid was obtained. When it was administered orally, the yield was decreased to about two-thirds. After being purified by esterification, the acid gave a *p*-phenylphenacyl ester of m. p. 99.5–101°. From the data of elemental analysis, it corresponded to the bis-*p*-phenylphenacyl ester of C₉ dicarboxylic acid.



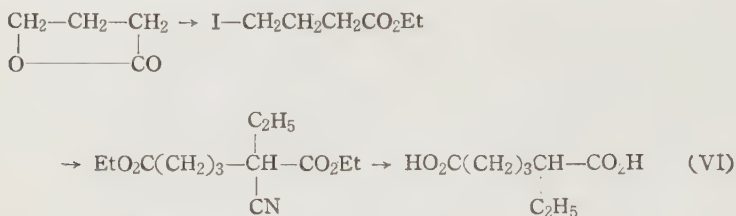
(3) Ethyl group at β -position. 3-Ethylanthanic acid (IV) was prepared and a total of 9.5 g. was injected into a rabbit. From the urine, 2.3 g. of unchanged monobasic acid, and 1.8 g. of viscous mass was obtained. Purified as above, the latter gave a *p*-phenylphenacyl ester of m. p. 58–60°. Elemental analysis indicated it as the bis-*p*-phenylphenacyl ester of C₉-dibasic acid.



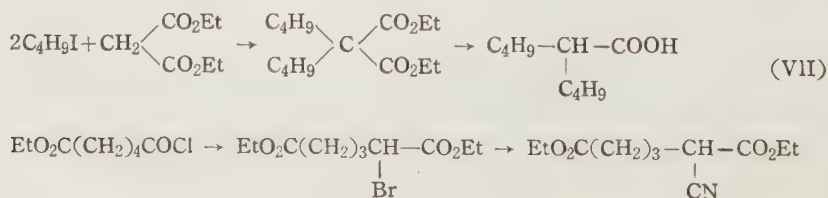
(4) Ethyl group at α -position. As an example of these compounds, we injected 2-ethylhexanol (V) into a rabbit subcutaneously. After a total of 11 g. was administered during ten days, we obtained from the urine a volatile neutral portion and a volatile acid portion.

The latter was proved to be 2-ethylcaproic acid, for its *p*-toluidide melted at 107° and showed no depression of the melting point when fused with the same derivative of the synthetic 2-ethylcaproic acid (m. p. 107°).

From the residue, 0.9 g. of non-volatile viscous substance was isolated, which, after purification as above, gave a *p*-phenylphenacyl ester of m. p. 123–5°. Supposing it to be the bis-*p*-phenylphenacyl ester of 2-ethyladipic acid, we synthesized this substance for identification. Though the bis-*p*-phenylphenacyl ester of 2-ethyladipic acid (VI) melted at 118–120°, the mixed melting point of these two esters lay between those of the two, and so we assumed both to be identical.



(5) α -Substituted symmetric side chains. 2-Butylcaproic acid (VII) was prepared and 10 g. of it was administered to a rabbit. From the urine 1.7 g. of volatile acid was obtained, which gave a *p*-toluidide of m. p. 115°, and was identified as the unchanged 2-butylcaproic acid. Then we isolated 2.2 g. of non-volatile acid as a viscous substance, which after purification gave *p*-bromophenacyl ester of m. p. 61–62.5°. The data of elemental analyses indicated it to be a C₁₀-dicarboxylic acid. We then synthesized 2-butyladipic acid (VIII) as follows:



carboxyl affects ω -oxidation as favorably as that at β -position has hitherto not been pointed out.

We take pleasure in acknowledging our indebtedness to Prof. T. Hoshino of Tokyo Institute of Technology for supplying octanol and 2-ethylhexanol.

We are also indebted to Miss. S. Suzuki and Miss. S. Irie for elemental analyses and to Miss. E. Kashiwabara for her kind assistances.

EXPERIMENTAL.

(1) Methyl group at β -position. 12.3 g. of 3-methylpelargonic acid, b. p.₁₂ 144–5°, was administered during 10 days to a rabbit by approximately the same manner as described in the 1st Report. From the collected urine, following two portions were obtained: (a) b. p.₁ 100°, 1.5 g.; and (b) b. p._{0.1} 150–170°, 3.8 g. The former fraction soon solidified and became colorless needles, m. p. 121°, which was identified as benzoic acid. The latter fraction (b) was a viscous, yellowish oil, and by treatment with diazomethane, 1.3 g. of colorless liquid, b. p._{1.5} 116–120°, n_D^{11} : 1.4489, was obtained. After saponification with 10 per cent alcoholic potash, 0.5 g. of viscous, colorless liquid, b. p._{0.2} 166–170°, n_D^{19} : 1.4670, was isolated. *p*-Bromophenacyl ester: colorless needles, m. p. 100–101.5°. Calcd. for $C_{26}H_{28}O_6Br_2$: C 52.35; H 4.73. Found: C 52.27; H 4.56.

Synthesis of 3-methylazelaic acid.

Starting from cyclohexanone, 1.7 g. of 3-methyl- Δ^{2-3} azelaic acid, b. p._{1.2} 190–1°, n_D^{22} : 1.4801, was prepared according to the method of Kuhn (15). (Kuhn's sample gave b. p._{0.002} 172–4° (bath-temp.), n_D^{20} : 1.4800). 0.8 g. of this substance was dissolved in 60 ml. of glacial acetic acid, reduced with hydrogen in the presence of palladium carbon. The acid obtained after reduction weighed 0.5 g, b. p._{0.5} 174°, m. p. 43°, n_D^{23} : 1.4565. (The same substance prepared by a different method melted at 43–4°, (16).)

Bis-*p*-bromophenacyl ester: colorless needles, m. p. 101-2°.

Calcd. for $C_{26}H_{28}O_6Br_2$: C 52.35; H 4.73. Found: C 52.17; H 4.74.

(2) Methyl group at α -position. 10 g. of 2-methylcaprylic acid, b. p.₂₃ 137-142°, n_D^{26} : 1.4287, was prepared and administered to a rabbit. From the urine, by the usual procedure, 1.5 g. of benzoic acid and 1.08 g. of viscous yellowish mass, b. p._{0.5} 150-174°, were obtained. 1.5 g. of the latter fraction was esterified with diazomethane and the methyl ester, b. p.₁₁ 128-132°, n_D^{20} : 1.4450, was saponified, after which the free acid, b. p.₁ 160-170°, n_D^{20} : 1.4602, was isolated.

p-Phenylphenacyl ester: colorless needles, m. p. 99.5-101°.

Calcd. for $C_{44}H_{36}O_6$: C 77.07; H 6.25. Found: C 76.27; H 6.26.

(3) Ethyl group at β -position. 9.5 g. of 3-ethylenanthic acid, b. p.₂₅ 145-147°, was administered to a rabbit. From the urine, following three portions were separated: (a) b. p.₂₁ 131-140°; 1.1 g., (b) b. p.₂₁ 140-2°; 2.3 g., (c) b. p._{0.5} 160°- b. p._{0.03} 160°; 1.8 g. From the last fraction, 1.2 g. of methyl ester, b. p.₁₅ 132-8°; n_D^{16} : 1.4460, was prepared, which after saponification gave 0.8 g. of free acid, b. p._{0.04} 170°.

p-Phenylphenacyl ester: Colorless needles, m. p. 58-60°.

Calcd. for $C_{44}H_{36}O_6$: C 77.07; H 6.25. Found: C 77.21; H 6.16.

(4) Ethyl group at α -position. 11.0 g. of 2-ethylhexanol, b. p. 184°, was injected in 1 g. portions twice a day. From the volatile portion of the urine, 1.3 g. of acid fraction, b. p.₂₄ 130-132°, and 1.2 g. of neutral portion, b. p.₁₆ 84°, were obtained. The former fraction gave a *p*-toluidide of colorless needles, melting at 107°, which showed no depression of the melting point when fused with *p*-toluidide of 2-ethylcaproic acid, prepared by the oxidation of 2-ethylhexanol, m. p. 107°. From the non-volatile portion of the urine, 0.9 g. of viscous, yellowish liquid, b. p._{0.2} 160-170°, was obtained. This was esterified with diazomethane and 0.5 g. of methyl ester, b. p.₁₄ 123-130°, n_D^{21} : 1.4410, was isolated.

p-Phenylphenacyl ester: colorless powder, m. p. 123-5°.

Calcd. for $C_{36}H_{34}O_6$: C 76.86; H 6.09. Found: C 76.72; H 6.28

Synthesis of 2-ethyladipic acid.

1.4 g. of sodium was dissolved in absolute alcohol, 15 g. of ethyl 2-cyanobutyrate and 12 g. of ethyl 4-iodobutyrate were added and refluxed for ten hours. After treatment in the usual manner, 8 g. of colorless liquid, b. p._{0.4} 134–6°, was isolated. 4 g. of this substance was saponified by heating with 10 ml. of conc. hydrochloric acid in a sealed tube at 170° for five hours. From the dark brown reaction product, 1.2 g. of viscous, colorless liquid, b. p._{0.1} 166°, was obtained, which after standing overnight in a refrigerator, solidified to crystals of m. p. 48–9° (the same sample prepared by a different method melted at 48–50° (17)).

Calcd. for $C_8H_{14}O_4$: C 55.17; H 8.05. Found: C 55.38; H 8.21.

Bis-*p*-phenylphenacyl ester: colorless powder, m. p. 118–120°.

Calcd. for $C_{36}H_{34}O_6$: C 76.86; H 6.09. Found: C 76.62; H 6.35.

(5) α -Substituted symmetric side chains. 10.0 g. of 2-butylcaproic acid, b. p.₂₂ 158°, was administered to a rabbit. From the volatile portion of the urine, 1.7 g. of an acid, b. p.₂₁ 140–151°, was obtained, *p*-toluidide of which was colorless needles, melting at 115°, and there was no depression of the melting point when fused with the *p*-toluidide of 2-butylcaproic acid. From the non-volatile portion 1.3 g. of benzoic acid and 2.2 g. of crude dibasic acid, b. p._{0.2} 165–185°, were separated. The latter fraction was esterified with diazomethane and 1.0 g. of methylester, b. p.₁₂ 144–8°, n_D^{20} : 1.4435, was obtained. After saponification, 0.55 g. of free acid, b. p._{0.5} 176°, was obtained.

p-Bromophenacyl ester: colorless needles, m. p. 61–62.5°.

Calcd. for $C_{25}H_{28}O_6Br_2$: C 52.35; H 4.73. Found: C 51.79; H 4.59.

Synthesis of 2-butyladipic acid.

Diethyl 2-bromoadipate—40 g. of dried bromine was added to 50 g. of ω -carbethoxypentoyl chloride, b. p.₁₁ 116°, in the presence of a small amount of red phosphorus and heated on a water bath for four hours. After the evolution of hydrobromic acid gas ceased, 50 ml. of absolute alcohol was added slowly from a separatory funnel.

From the reaction product, 46.5 g. of neutral, colorless liquid, b. p.₈ 145–7°, was isolated. After rectification, 24 g. of a substance, b. p.₁₀ 150–2°, n_D^{25} : 1.4632, was obtained.

Calcd. for $C_{10}H_{17}O_4Br$: C 42.70; H 6.05. Found: C 42.75; H 6.14.

Diethyl 2-cyano adipate—30 g. of diethyl 2-bromoadipate was dissolved in 180 ml. of methanol, 10 g. of powdered KCN was added and refluxed for five hours. 5 g. of KCN was further added and refluxing was continued for eight hours. After evaporation of methanol, about 100 ml. of water was added and the mixture was extracted continuously with ether. The crude ethereal extract was esterified with absolute alcohol and conc. sulfuric acid, after which 7.7 g. of neutral, colorless liquid, b. p.₁₂ 170–172, n_D^{20} : 1.4408, was obtained.

Calcd. for $C_{11}H_{17}O_4N$: N 6.17. Found: N 6.08.

2-Butyladipic acid—2.0 g. of sodium was dissolved in absolute alcohol, 10 g. of diethyl 2-cyano adipate and 15 g. of n-butyl iodide were added and refluxed for ten hours. From the reaction mixture, 4.5 g. of neutral, colorless liquid, b. p._{1.5} 153–5°, n_D^{20} : 1.4480, was obtained. 3 g. of this substance, probably diethyl 2-cyano-2-butyladipate, was heated with conc. hydrochloric acid in a sealed tube for five hours at 160°. From the reaction mass, 0.95 g. of colorless liquid, b. p._{0.25} 166–172°, was obtained.

Another method:—1 g. of sodium was dissolved in absolute alcohol, 8 g. of diethyl butylmalonate, b. p.₁₉ 125–7°, and 9 g. of ethyl 4-iodobutyrate were added and refluxed for ten hours. From the reaction mass, 2.3 g. of colorless liquid, b. p._{1.2} 145–8°, n_D^{22} : 1.4385, was obtained. After saponification with 10 per cent alcoholic potash and decarboxylation by heating, 0.2 g. of colorless acid, b. p.₁₂ 220–250° (bath-temp.), n_D^{21} : 1.4520, was isolated.

Bis-*p*-bromophenacyl ester: colorless needles, m. p. 59.5–61°.

Calcd. for $C_{26}H_{28}O_6Br_2$: C 52.35; H 4.73. Found: C 52.44; H 4.50.

(6) β -Substituted symmetric side chains. 9.0 g. of 3-propylcaproic acid, b. p.₁₂ 132–5°, was administered to a rabbit. From the volatile portion of the urine, 1.1 g. of an acid, b. p.₁₄ 132–5°, was ob-

tained, p-toluidide of which, colorless needles, melting at 73° , showed no depression of the melting point when fused with the same derivative of 3-propylcaproic acid.

Calcd. for $C_{16}H_{25}ON$: C 77.73; H 10.12. Found: C 77.95; H 10.10.

Non-volatile ethereal extract was treated with diazomethane and separated into following portions by fractional distillation: (a) b. p.₂₀ $100-120^{\circ}$; 0.5 g., (b) b. p.₁₂ $100-130^{\circ}$; 0.4 g., (c) b. p.₁₂ 142° ; 1.2 g., and (d) b. p.₁ $140-150^{\circ}$; 0.9 g. The fraction (c), supposed to be a dimethyl ester of C_9 -dibasic acid, was saponified, after which 0.6 g. of free acid, b. p._{0.7} $170-175^{\circ}$, was obtained.

Calcd. for $C_9H_{16}O_4$: C 57.44; H 8.51. Found: C 57.60; H 8.76.

REFERENCES.

- (1) Asano, M. & Yamakawa, T., J. Biochem., **37**, 321 (1950).
- (2) Asano, M. et al., Igaku Sōron, **1**, 41, 42 (1945), J. Pharm. Soc., Japan, **69**, 126, 129 (1949).
- (3) Stanley, W. M., Roger Adams, *et al.*, J. Pharmacol., **45**, 121 (1932).
- (4) Anderson, R. J., Zechmeister's "Fortschritte der Chemie organischer Naturstoffe" III, 145 (1939).
- (5) Chargaff, E., Z. physiol. Chem., **201**, 191 (1932); **218**, 223 (1933).
Asano, M. & Takahashi, H., J. Pharm. Soc. Japan., **65**, 140 (1945).
Takahashi, H., J. Pharm. Soc. Japan., **68**, 35 (1948).
- (6) Nozoe, T., Unpublished.
- (7) Turner, K., Biochem. J., **25**, 49 (1931).
- (8) Sabin, Physiol. Rev., **12**, 141 (1932).
- (9) Velick, S. F., J. Biol. Chem., **152**, 533 (1944).
- (10) Kay, H. D. & Raper, H. S., Biochem. J., **18**, 151 (1924).
- (11) Kuhn, R. & Livada, K., Z. physiol. Chem., **220**, 235 (1933).
- (12) Verkade, P. E. *et al.*, Biochem. J., **28**, 31 (1934); Z. physiol. Chem., **225**, 230 (1933).
- (13) Flaschenträger, B. *et al.*, Z. physiol. Chem., **238**, 221 (1936).
- (14) Kuhn, R. & Löw, I., Z. physiol. Chem., **259**, 182 (1939).
- (15) Kuhn, R., Köhler, Fr. & Köhler, L., Z. physiol. Chem., **242**, 187 (1936).
- (16) Freer & Perkin, J. Chem. Soc., **53**, 218.
- (17) Lean & Lees, J. Chem. Soc., **71**, 1067; Mellor, *ibid.*, **79**, 131, Best & Thorp, *ibid.*, **95**, 713.

THE FATE OF BRANCHED-CHAIN FATTY ACIDS IN ANIMAL BODY. III. BIOLOGICAL OXIDATION OF BRANCHED-CHAIN FATTY ACIDS IN RE- LATIONSHIP TO OPTICAL ACTIVITY.*

By

SHICHIRO AKIYA and TAMIO YAMAKAWA.

*(From the 7th Department of the Institute for Infectious
Diseases, University of Tokyo).*

(Received for publication June 6, 1950).

In the previous paper of this series (1), Asano and Yamakawa mentioned that ω -oxidation of branched-chain fatty acids, which possessed asymmetric carbon atom in β -position to carboxyl group, was carried out without regard to their optical activity, namely, when administered to rabbits, both l-rhodinic and d-citronellic acids were excreted as almost optically pure dihydro-Hildebrandt acid. Subsequently, several synthetic branched-chain fatty acids were given to rabbits and their mode of breakdown was studied (2). Although optically inactive materials were administered in those cases, slight optical rotations were noticed in many cases. These values of optical rotations were, however, so extremely slight that it required considerably high concentration of sample solutions necessary for determination, hence we repeated experiments several times and attempted to secure a large amount of final products until reliable results could be obtained.

As shown in Table I, synthetic substances in the first column were given to rabbits; the collected urine were acidified and concentrated under reduced pressure. By this procedure, unchanged monocarboxylic acids appeared in the distillate, possessing specific rotations as

* This was previously published in Japanese in J. Pharm. Soc. Japan; 70, 477 (1950).

TABLE I.

Substances administered	Specific rotations of substances excreted.	
	Monobasic acid.	Dibasic acid.
I) 2-Methylcaprylic acid $\begin{array}{c} \text{CH}_3 \\ \\ \text{C}_6\text{H}_{13}\text{CH}-\text{COOH} \end{array}$	+1.2 (7.92%, acetone)	—
II) 3-Methylpelargonic acid $\begin{array}{c} \text{CH}_3 \\ \\ \text{C}_6\text{H}_{13}\text{CH}-\text{CH}_2\text{COOH} \end{array}$	+1.2 (15.6%, acetone)	+2.31 (7.36%, acetone) +3.28 (6.57%, acetone)* +1.94 (26.4%, acetone)*
III) 2-Ethylcaproic acid $\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{C}_4\text{H}_9\text{CH}-\text{COOH} \end{array}$	+0.58 (30.9%, acetone)	±0.0 (25.3%, acetone)*
IV) 2-Ethylhexanol $\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{C}_4\text{H}_9\text{CH}-\text{CH}_2\text{OH} \end{array}$	+1.73 (13.8%, ethanol) +1.48 (6.07%, acetone)	+0.8 (7.60%, acetone)*
V) 3-Ethylenanthic acid $\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{C}_4\text{H}_9\text{CH}-\text{CH}_2\text{COOH} \end{array}$	±0.0 (7.37%, acetone) ±0.0 (12.4%, ethanol)	±0.0 (3.45%, ethanol) -0.7 (15.3%, acetone)*
VI) Dibutylacetic acid $\begin{array}{c} \text{C}_4\text{H}_9 \\ \\ \text{C}_4\text{H}_9\text{CH}-\text{COOH} \end{array}$	—	±0.0 (1.5%, ethanol) ±0.0 (1.0%, ethanol)*
VII) Dipropylpropionic acid $\begin{array}{c} \text{C}_3\text{H}_7 \\ \\ \text{C}_3\text{H}_7\text{CH}-\text{CH}_2\text{COOH} \end{array}$	—	±0.0 (4.17%, methanol)* +0.47 (11.5%, ethanol)*

* = estimated in the form of dimethyl ester.

shown in the second column. In cases I, II and III, the excreted substances had the same structural formulae as the original ones, but were optically active. Therefore, it would seem that, during the passage of these acids through the animal body, a certain process, e. g., absorption, breakdown, excretion, *etc.*, is carried out one-sidedly.

In case of IV, the substance obtained by this treatment was subjected to the oxidation of primary alcohol to carboxyl and, therefore, its optical activity could have been produced either in this process of oxidation or during its passage through animal body similar to above cases, or by a combination of these two. Nevertheless, the observed value was markedly higher than that in the case of III, in which the substance administered was 2-ethylcaproic acid, the end-product of case IV. This fact suggests that the appearance of marked optical activity in case IV is due to the oxidation of alcohol to carboxyl.

When the residual non-volatile portion of urine was extracted with ether, dibasic acids were obtained, possessing optical activity as shown in the third column. In cases I, II, III and V, the substances administered were subjected to biological oxidation of terminal methyl into carboxyl group and, moreover, suffered optical changes similar to the cases of monobasic acids. Therefore, it is as yet undetermined to which process optical activities shown in the third column are responsible.

The appearance of optical activities of dibasic acids in cases I to V, even if the oxidation proceeds one-sidedly, cannot be termed as asymmetric synthesis in the strict sense of the word (3).

However, in the case of VI, the original substance contained no symmetric carbon atom and it became asymmetric as a result of ω -oxidation; therefore, a marked optical rotation was expected. However, the dibasic acid obtained, 2-butyladipic acid, was optically inactive. Since, in this case, the newly formed asymmetric carbon atom lies in the α -position to carboxyl group, and it was exposed to high temperature in the course of purification, it was suspected to have been racemized by tautomerization. In order to avoid this risk, 3,3-dipropylpropionic acid was administered to rabbits. The dibasic acid obtained, 3-propyladipic acid, had a slight optical activity. Since it is not clear whether this was formed during biological oxidation of methyl to carboxyl group, or in other processes of passage, it could hardly be assumed that the optical activity of this sort was

exclusively due to ω -oxidation.

As mentioned above, the values of optical activity formed in the organism seemed to be influenced by the molecular shape and dimension of the substance administered, and therefore, the regularity of these optical changes could not be determined. However, it is worthy of note that all but one of the optical rotations of substances excreted were dextrorotatory.

BIBLIOGRAPHY.

- (1) Asano, M., & Yamakawa, T., J. Biochem., **37**, 321 (1950).
- (2) Asano, M., & Yamakawa, T., J. Biochem., **37**, 329 (1950).
- (3) McKenzie, A., Ergeb. Enzymforsch., **5**, 49 (1936).

THE FATE OF BRANCHED-CHAIN FATTY ACIDS IN ANIMAL BODY. IV. A CONTRIBUTION TO THE MODE OF BIOLOGICAL BREAKDOWN OF NATURALLY OCCURRING FATTY ACIDS.*

BY

TAMIO YAMAKAWA.

*(From the 7th Department of the Institute for Infectious
Diseases, University of Tokyo.)*

(Received for publication June 6, 1950.)

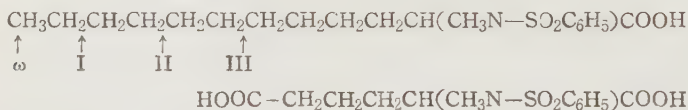
It has been shown in the preceding paper (1), that all of the branched-chain fatty acids containing C_8 to C_{10} suffered ω -oxidation in animal body and were excreted as dibasic acids. However, the process of ω -oxidation has been shown to be restricted within C_8 to C_{11} by the experiments with straight-chain fatty acids of Verkade (2) and Flaschenträger (3). For that reason, many authors are apt to take a view that ω -oxidation plays little part in the biological oxidation of naturally occurring fatty acids. Although at present, the classical β -oxidation theory of Knoop (4) is not accepted as in former times, yet it holds its firm authority. This theory is based chiefly on the experiments of feeding phenyl fatty acids, liver perfusion and enzyme experiment with lower fatty acids and the observation of acetone body formation. Nevertheless, experimental evidences, whether or not it is applicable to naturally occurring higher fatty acids of C_{16} to C_{18} series, are still almost lacking. Only the experiment of Schönheimer and Rittenberg (5) in which deuteropalmitic acid was isolated from the subcutaneous

* This study was published in Japanese. (J. Pharm. Soc., Japan; 70, 633 (1950)).

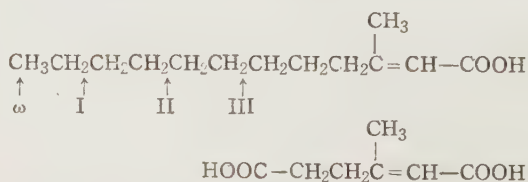
adipose tissue of rats fed with deuterostearic acid, indicated the splitting of 2 C-fragment in the natural series.

This difficulty of demonstrating the evidence of β -oxidation forced investigators to seek other pathways of degradation of naturally occurring fatty acids. Thus, ω -oxidation, multiple alternate oxidation and a number of other theories have been forwarded.

The metabolism of higher branched-chain fatty acid has already been studied by Flaschenträger (6) and Kuhn (7), and more recently by Keil (8). Flaschenträger fed α -methylbenzenesulfaminolauric acid to a dog, isolated from its urine α -methylbenzenesulfaminoadipic acid and illustrated this process by the explanation that ω -oxidation took place at first, which was followed by three successive β -oxidation, as shown in the following schema.



Kuhn, et al. administered β -methyl- β -nonylacrylic acid to a rabbit and obtained β -methyl- $\Delta^{\alpha,\beta}$ -adipic acid from its urine. He also attempted the explanation similar to that of Flaschenträger, as follows:

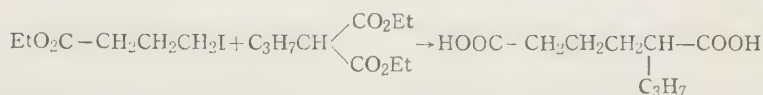


In this case, the fatty acid administered contains 13 C-atoms, hence is still lower than the naturally occurring C_{16} to C_{18} series. On that account, Kuhn synthesized β -methylstearic acid (C_{19}) and fed to a rabbit, but he could isolate only a trace of oil, which was left undetermined.

In the present experiments, two branched-chain fatty acids, C_{16} and C_{17} , were prepared and administered to rabbits, and the chemical

composition of the products obtained from the urine was determined.

After administration of 2-propylmyristic acid (C_{17}) and treatment in the usual manner, an acid (tentatively called as C_{17} -diacid) was isolated from the urine. It gave a *p*-phenylphenacyl ester melting at 108° . The analytical results of this substance were in agreement with the composition of bis-*p*-phenylphenacyl ester of C_8 , C_9 or C_{10} -dibasic acid. Supposing the C_{17} -diacid to be 2-propyladipic acid (C_9), this was prepared in the following manner:



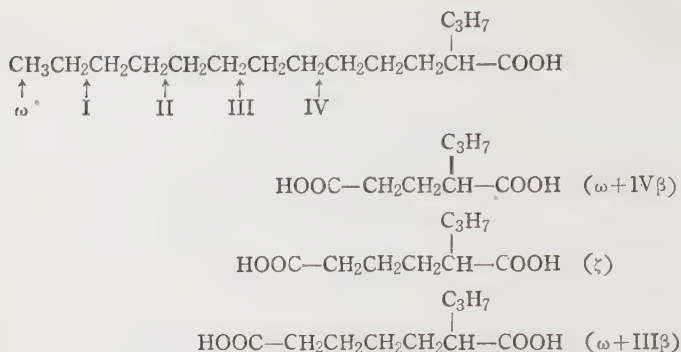
The bis-*p*-phenylphenacyl ester of this synthetic dibasic acid melted at 108° , and showed no depression of the melting point when fused with the same derivative of the C_{17} -diacid.

Consequently, it was suggested that in this case, too, ω -oxidation took place at first, and then β -oxidation followed successively four times, thereby eight-carbon fragments being spilt off. Nevertheless, another possible manner of degradation can be considered. In this and preceding two cases of the german authors, number of carbon atoms in the main chain of the excreted dibasic acid is six; namely, in every case, modified "adipic acid" is obtained.

Therefore, a mode of degradation is conceivable in which the cleavage takes place at the sixth position (ζ -position) to the carboxyl group present from the beginning, and then the terminal methyl group is oxidized.

In order to demonstrate this hypothesis of ζ -oxidation, it was considered to be verified, if C_{17} -diacid (2-propyladipic acid) could be isolated from the urine of a rabbit administered a fatty acid, which differed from 2-propylmyristic acid in the main carbon chain by odd numbers.

For this purpose, 2-propyltridecanoic acid (C_{16}) was prepared and administered to a rabbit.



After similar treatment, small amount of an acid (C_{16} -diacid) was isolated, the *p*-phenylphenacyl ester of which melted at $108\text{--}109^\circ$, and elemental analysis showed that it also corresponded to the *b*'s-*p*-phenylphenacyl ester of $\text{C}_8\text{--C}_{10}$ dibasic acid.

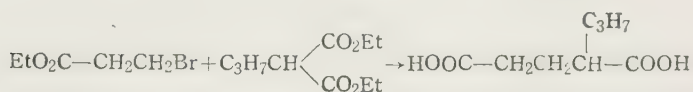
TABLE I.

	b. p.	Bis- <i>p</i> -phenylphenacyl ester		
		m. p.	C %	H %
C_{17} -Diacid	$180\text{--}200^\circ/0.4$ (bath-temp.) semisolid.	108°	76.82	6.22
C_{16} -Diacid	$160\text{--}4^\circ/0.1$ semisolid.	$108\text{--}9^\circ$	76.83	6.30
2-Propylglutaric acid (C_8)	$166\text{--}7^\circ/0.3$ m. p. 68°	139°	76.67 76.86*	5.93 6.06*
2-Propyladipic acid (C_9)	$157\text{--}8^\circ/0.1$ m. p. 59°	108°	77.07 77.08*	6.69 6.25*
2-Propylpimelic acid (C_{10})	$187\text{--}8^\circ/0.15$	108.5°	77.65 77.29*	6.77 6.44*

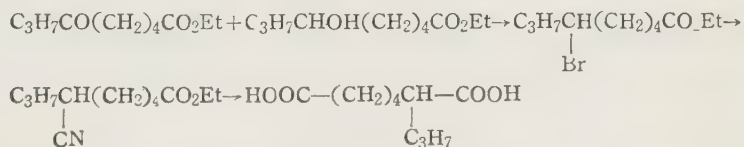
* = calculated value.

From the properties shown in Table I, the excreted C_{16} -diacid was thought to be identical with C_{17} -diacid (2-propyladipic acid), in which case, experimental evidence of ζ -oxidation would be established.

However, the mixed melting point of *p*-phenylphenacyl esters of both C_{16} - and C_{17} -diacids fell to 88°C . and the experimental demonstration of ζ -oxidation resulted in a failure. Therefore, it became necessary to determine the nature of the C_{16} -diacid. From the boiling point and data of elemental analysis, it was supposed to be 2-propylglutaric (C_8) or 2-propylpimelic (C_{10}) acid. At first 2-propylglutaric acid was synthesized in the following way :



However, the bis-*p*-phenylphenacyl ester of this substance melted at 139° , indicating it to be different from the C_{16} -diacid, although the mixed melting point of these two substances lay at about 120° , showing the C_{16} -diacid to contain some substances other than 2-propylglutaric acid. The amount of the sample was so small that the separation of constituents in the C_{16} -diacid seemed extremely difficult and laborious, and could not be carried out to completion by fractional distillation. Therefore, 2-propylpimelic acid, the most likely other half, was prepared.



The melting point of bis-*p*-phenylphenacyl ester of this substance was 108° , which corresponded not only in the melting point but also in the analytical values of the same derivative of the C_{16} -diacid within experimental errors. Nevertheless, the mixed melting point of these two lay at 98° , showing both were not identical.

This apparently inexplicable results became clear, when the mixed melting point curve of bis *p*-phenylphenacyl esters of both 2-propylglutaric and 2-propylpimelic acids was plotted (Fig. 1). Another point on this curve which showed m. p. 108° lay at the spot

where $C_8:C_{10}=7:3$. There was no depression of the melting point when the mixed substance, $C_8:C_{10}=7:3$, was fused with the derivative of the C_{16} -diacid. Accordingly, it was decided that the C_{16} -diacid consisted of 7 parts of 2-propylglutaric acid and 3 parts of 2-propylpimelic acid. This method of demonstration was somewhat complicated, since the mixed melting point of a substance with a mixture was determined. There was some doubt whether such a method was reliable or not, hence the mixed melting point curve of bis-p-phenylphenacyl ester of 2-propylglutaric (C_8) and 2-propyladipic acid (C_9) was plotted. (Fig. 2). Another point on this curve showing m.p. 108° lay at the spot where $C_8:C_9=5:5$. The mixed melting point of the C_{16} -diacid with the mixture of $C_8:C_9=5:5$, lay at 123° , by which it was proved that this method was valid.

Fig. 1.

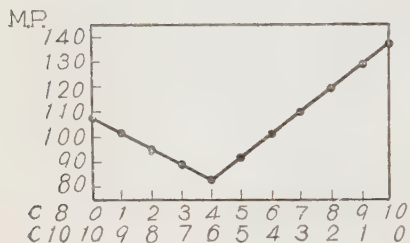
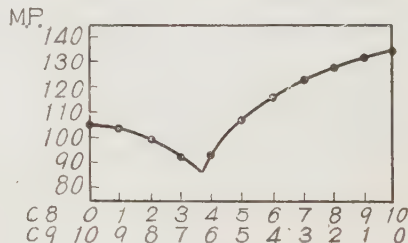


Fig. 2.



COMMENT.

The studies of the metabolism of fatty acids were hitherto confined to lower fatty acids. Experiments with naturally occurring higher fatty acids were almost lacking. Hence, little was known about their mode of oxidation.

According to the present experiments with higher branched-chain fatty acids, although they cannot be regarded in the same light with the straight-chain fatty acids, the naturally occurring fatty acid suffered ω -oxidation in the first place, then the carbon chain was split by 2 C-fragment from the newly formed carboxyl group.

I wish to express my gratitude to the late Prof. M. Asano, Prof. S. Akiya, Dr. T. Ukita and Dr. H. Yoshikawa for their kindness and helpful suggestions in carrying out this work.

I am also indebted to Miss. S. Suzuki, Miss. S. Irie and Mr. B. Kurihara for elemental analysis.

EXPERIMENTAL.

Synthesis of 2-propylmyristic acid:—14 gm. of sodium was dissolved in absolute alcohol, 65 g. of ethyl cyanoacetate and 100 g. of n-propyl iodide were added and refluxed for eight hours. After treatment in the usual manner, 33 g. of ethyl n-propylcyanoacetate, b.p. 220–227°, was isolated.

5.6 g. of sodium was dissolved in absolute alcohol, 33 g. of ethyl n-propylcyanoacetate and 71 g. of dodecyl iodide were added and refluxed for ten hours. After treatment in the usual manner, 36 g. of ethyl n-propyl-n-dodecylcyanoacetate, b.p._{1.6} 165°, n_D^{25} : 1.4432, was obtained.

30 g. of this substance was saponified by heating with 30 ml. of HCl conc. in a sealed tube at 160° for three hours. From the dark brown reaction product, 15.5 g. of colorless liquid, b.p._{1.5} 167–9°, n_D^{25} : 1.4460, was isolated, which, after standing overnight, became a solid, m.p. 24.5–26.2°. *p*-Toluidide: colorless needles, m.p. 96°.

Calcd. for $C_{24}H_{41}ON$: C 80.22; 11.42. Found: C 80.40; H 11.63.

Animal experiment with 2-propylmyristic acid:—9.0 g. of 2-propylmyristic acid was administered to a rabbit during ten days. The collected urine was acidified with hydrochloric acid, concentrated under reduced pressure to about 300 ml. and extracted continuously with ether. 1.5 g. of the crude acid portion of the ethereal extract was treated with diazomethane. 1.6 g. of this neutral reaction product was attempted to be rectified in vacuo, but obstinate foaming inhibited this procedure. Hence, this methylated substance was saponified with alcoholic potash and the free acid was fractionated into the following two portion: (a) b.p.₁ 130–160°, 0.1 g.; (b)

b. p._{0.4} 180–200°, 0.3 g.; (bath temperature). Fraction (b) was named tentatively as C₁₇-diacid. *p*-Phenylphenacyl ester of the C₁₇-diacid: colorless powder, m. p. 108°.

Calcd. for C₃₇H₃₆O₆: C 77.08; H 6.25. Found; C 76.82; H 6.22.

Synthesis of 2-propyladipic acid:—0.2 g. of sodium was dissolved in 50 ml. of absolute alcohol, 1.5 g. of diethyl *n*-propylmalonate and 1.7 g. of ethyl 4-iodobutyrate were added and refluxed for eight hours. After treatment in the usual manner, 0.4 g. of a neutral liquid, b. p._{0.1} 130–135°, was isolated. After saponification with 10 per cent alcoholic potash, colorless crystals (tricarboxylic acid) were isolated, which, after decarboxylation by heating, was converted to a dibasic acid, b. p._{0.3} 166–7°, m. p. 59°.

Bis-*p*-phenylphenacyl ester: colorless powder, m. p. 108°.

Calcd. for C₃₇H₃₆O₆: C 77.08; H 6.25. Found: C 77.07; H 6.69.

Synthesis of 2-propyltridecanoic acid:—Undecylenic acid, b. p.₁₈ 167°, was dissolved in methanol and reduced with hydrogen in the presence of platinum oxide. The acid obtained after reduction was esterified with methanol and conc. sulfuric acid. 80 g. of methyl undecanoate, b. p.₂₂ 130–5°, was reduced in 85 atm. of hydrogen in the presence of 7 g. of copper chromite. After shaking for eight hours at 250–270°, the reaction product was boiled with sodium hydroxide and the acid portion was discarded. The neutral portion was fractionated and 41 g. of undecanol, b. p.₁₅ 135°, was obtained. After 30 g. of undecanol was treated with 23 g. of iodine and 2 g. of red phosphorus, 47.3 g. of undecyl iodide, b. p.₁₆ 152°, was isolated. 2.3 g. of sodium was dissolved in 200 ml. of anhydrous butanol, 20 g. of diethyl propylmalonate and 28 g. of undecyl iodide were added and refluxed for ten hours. After treatment in the usual manner, 26 g. of colorless liquid, b. p._{1.5} 190–208°, was obtained. After saponification with alcoholic potash and decarboxylation by heating, 12.3 g. of colorless liquid, b. p._{3.5} 180–183°, was obtained, which solidified showing m. p. 20–21°.

p-Toluidide: colorless needles, m. p. 90°.

Calcd. for $C_{23}H_{39}ON$: C 80.00; H 11.30; N 4.4. Found: C 80.40; H 11.93; N 4.35.

Animal experiment with 2-propyltridecanoic acid:—10.0 g. of 2-propyltridecanoic acid was administered to two rabbits. From the collected urine, by the usual procedure, 4.0 g. of crude acid portion was obtained. After treatment with diazomethane, 4.0 g. of crude neutral portion was obtained and fractionated as follows: (a) b. p.₁₆ 90–100°; 0.7 g.; (b) b. p.₁₆ 125–140°; (c) b. p.₁₆ 140°; (d) b. p._{0.5} 140–160°, 0.9 g. Fraction (a) was saponified and identified as benzoic acid. (b) and (c) were combined (0.4 g.) and saponified, after which 0.3 gm. of a free acid, b. p._{0.1} 160–4°, was obtained. (This was named tentatively as C_{16} -diacid.) *p*-Phenylphenacyl ester of the C_{16} -diacid: colorless powder, m. p. 108–9°.

Found: C 76.83; H 6.30.

Synthesis of 2-propylglutaric acid:—0.5 g. of sodium was dissolved in 30 ml. of absolute alcohol, 4 g. of diethyl propylmalonate and 3.6 g. of ethyl 3-bromopropionate were added and refluxed for eight hours. After treatment in the usual manner, 2.8 g. of neutral liquid, b. p.₁₉ 182–4°, $n_D^{23.5}$: 1.4360, was obtained. 2.0 g. of this substance was saponified with alcoholic potash and decarboxylated by heating, after which viscous acid, b. p._{0.5} 165°, $n_D^{24.5}$: 1.4493, was obtained, which after standing overnight in the refrigerator with petroleum ether, became a solid of m. p. 68°.

Bis-*p*-phenylphenacyl ester: colorless powder, m. p. 139°.

Calcd. for $C_{36}H_{34}O_6$: C 76.86; H 6.06. Found: C 76.67; H 5.93.

Synthesis of 2-propylpimelic acid:—6-ketnonanoic acid:—A Grignard reagent was prepared from 24 g. of *n*-propyl iodide and 4.9 g. of Mg in 100 ml. of ether. To the solution was added, during five minutes, 19.6 g. of $CdCl_2$, then the mixture was stirred under reflux. Ether was distilled off, 65 ml. of benzene was added and distillation was continued until an additional 25 ml. of distillate had been collected. 120 ml. of benzene was then added, the solution was heated with stirring to boiling and, without external heating, 24 g.

of ω -carbethoxy pentoylchloride in 30 ml. of benzene was added slowly. Heating under reflux with stirring was continued for ten minutes, at which time the stirrer was stopped by the mass of precipitate. After cooling overnight, the reaction product was treated with ice and HCl dil. and 13 g. of colorless neutral liquid, b. p.₃₀ 155°, was isolated. After saponification with alcoholic potash, 10 g. of a free acid, 6-ketononanoic acid, b. p._{0.1} 130–4°, was obtained. Semicarbazone: colorless needles, m. p. 150–1°.

Calcd. for $C_{10}H_{19}O_3N_3$: C 52.40; H 8.64. Found: C 52.52; H 8.45.

6 gm. of 6-ketononanoic acid was refluxed with absolute alcohol and conc. sulfuric acid, and 6.0 g. of ethyl 6-ketononanoate, b. p._{18.5} 145°, was obtained.

6-hydroxynonanoic acid:—5 g. of Al-isopropoxide was dissolved in 50 g. of isopropyl alcohol, and 6 g. of ethyl 6-ketononanoate was added slowly and heated under reflux, until a negative acetone test was obtained (2 hours). After evaporation of isopropyl alcohol, the residue was treated with ice and HCl dil. and saponified with alcoholic potash, after which, 4.1 g. of 6-hydroxynonanoic acid, b. p._{0.15} 143–7°, n_D^{24} :1.4519, was obtained.

p-Phenylphenacyl ester: colorless needles, m. p. 79–80°.

Calcd. for $C_{23}H_{24}O_4$: 75.00; H 7.61. Found: C 75.44; H 7.81.

6-Bromononanoic acid:—4.0 g. of 6-hydroxynonanoic acid was dissolved in 10 ml. of glacial acetic acid and the ice-cold solution was saturated with hydrobromic acid gas. The saturated solution was heated on a boiling water-bath and this procedure was repeated three times. From the reaction mixture, 4.0 g. of acid, b. p._{0.07} 132–3°, n_D^{20} :1.4683, was obtained.

2-Propylpimelic acid:—3.1 g. of 6-bromononanoic acid was dissolved in 50 per cent alcohol, 5.0 g. of KCN was added and refluxed for twenty hours. Alcohol was evaporated, acidified with hydrochloric acid and extracted continuously with ether. 2.0 g. of viscous ethereal extract was saponified by heating with conc. HCl in a sealed tube at 170° for five hours. 0.6 g. of viscous acid, b. p._{0.15} 187–8°,

was obtained. Bis-p-phenylphenacyl ester: colorless needles, m.p. 108–108.5°.

Calcd. for $C_{38}H_{38}O_6$: C 77.29; H 6.44. Found: C 77.56; H 6.77.

REFERENCES.

- (1) Asano, M. & Yamakawa, T., J. Biochem., **37**, 329 (1950).
- (2) Verkade, P.E., Z. physiol. Chem. **225**, 230 (1934).
- (3) Flaschenträger, B. *et al.*, Z. physiol. Chem. **233**, 221 (1936).
- (4) Knoop, F., Beitr. chem. Physiol. Path., **6**, 150 (1905).
- (5) Schönheimer, R. & Rittenberg, D., J. Biol. Chem. **120**, 155 (1937).
- (6) Flaschenträger, B. *et al.*, Z. physiol. Chem. **225**, 157 (1934).
- (8) Kuhn, R., Köhler, Fr. & Köhler, L., Z. physiol. Chem., **242**, 187 (1936).
- (8) Keil, Chem. Abstr. **37**, 5943, 6728 (1943); Z. physiol. Chem., **274**, 175; **276**, 26 (1942); **282**, 137 (1947).

STUDIES ON ENZYMATIC ADAPTATION SUCCESSIVE ADAPTATION, WITH SPECIAL REFERENCE TO THE METABOLISM OF TRYPTOPHAN.*

BY

MASAMI SUDA, OSAMU HAYAISHI and YOSHIHARU ODA.

*(Department of Bacteriochemistry, Research Institute for Microbial
Diseases, Osaka University, Osaka).*

(Received for publication June 7, 1950).

Various investigations have been published since Karstroem concerning the phenomenon of microorganisms acquiring the corresponding enzymatic apparatus in response to their chemical environment.

Recent investigations made by Spiegelman (1) postulated the hypothesis that galactozymase and maltozymase in yeast cells are produced from non-enzymatic material by self-duplicating plasmagene (The Plasmagene Theory).

In 1943 Mirick (2) published that a soil organism which belongs to *Pseudomonas* sp. can produce adaptive enzymes which catalyze the oxidative breakdown of para-amino benzoic acid as well as ortho amino benzoid acid (anthranilic acid) when these substances are added to the medium previously and that the cells can also be adapted to anthranilic acid when tryptophan is substituted for anthranilic acid.

The authors took a hint from Mirick's results and performed investigation concerning the adaptive behavior of enzyme production

* The compendium of this article was announced at the Annual Meeting of the Society of Japanese Biochemists in April 1948 at Tokyo University and at the Symposium on Enzyme Chemistry in October 1948 at Tokyo University.

in microorganisms and the enzyme system of tryptophan metabolism.

In 1947 Stanier (3) reported that the oxidative breakdown of aromatic compounds such as benzoic-, mandelic-, para hydroxy benzoic-, phenyl acetic-acids and benzaldehyde is catalyzed by adaptive enzymes of *Pseudomonas* sp. He named the principle as "Simultaneous Adaptation" and made it a new technique for analyzing the dissimilatory process of such compounds as described above.

We have found that in *Pseudomonas* sp. tryptophan is oxidized via Kynurenin to anthranilic acid and the enzymes concerned are adaptively and successively produced. It was further established that anthranilic acid is oxidized through pyrocatechin and the disruption of benzene nucleus takes place.

We discovered these phenomena quite independently from Stanier and named these enzymatic adaptation as "Successive Adaptation", which seems to us more suitable because of its precise mechanism as described below.

EXPERIMENTAL

Material:

Several strains of *Pseudomonas* were isolated from the soil and pathological materials in our laboratory and among them were found a strain which broke down anthranilic acid. This strain however, unlike Mirick's strain, failed to produce the adaptive enzyme for the oxidative breakdown of para-amino benzoic acid.

Culture Media:

Carno-peptone (free of tryptophan)	1.00%
Beef extract	1.00%
NaCl	0.30%
KH ₂ PO ₄	0.05%

To the basic media of above composition were added 1-2 mg. of *l*-tryptophan or anthranilic acid per cc, pH. adjusted to 7.2 and autoclaved for sterilization.

Bacterial Suspension:

The cells cultured at 30° for 18-20 hours were harvested by centrifugation, washed twice with sterile distilled water and finally suspended in *M*/15 phosphate buffer of pH. 7.2.

Substrate:

Kanurenin used was granted us by Professor Y. Kotake of Wakayama Medical College. We wish to take this opportunity to express our heartfelt gratitude for his kindness in granting us such valuable preparation.

Method:

The oxidative breakdown of the substrates was pursued by measuring the oxygen consumption, using the manometric technique of Warburg. The substrates were made into solutions of *M*/100, and 0.2 ml. were used per vessel. The gas chamber contained air, while the water bath was regulated to 30°. The quantity of cells in the suspensions was measured with a nephelometer. Carbon dioxide output was calculated from the respiration quotient.

Results:

Suspensions of cells harvested from cultures containing *l*-tryptophan readily oxidized *l*-tryptophan (Tr.), *l*-kynurenin (Ky.) and anthranilic acid (Aa.) (full lines in Fig. 1), while suspensions of cells grown on the basic medium hardly oxidized any of them (dotted lines in Fig. 1). The Pc. curve in Fig. 1 will be referred to later.

Kynurenin which was discovered by Professor Kotake was found to be the intermediate product via which tryptophan is dissimilated into anthranilic acid. Further breakdown of anthranilic acid however, had not been clarified. At this point we found that our strain of *Pseudomonas* dissimilated tryptophan *via* the established route, and further more, that when this strain is allowed to adapt itself to anthranilic acid, a compound of lower metabolic order, it loses the power to breakdown tryptophan and kynurenin. Starting from these facts we succeeded in tracing the metabolic pathway below anthranilic acid in the following manner:

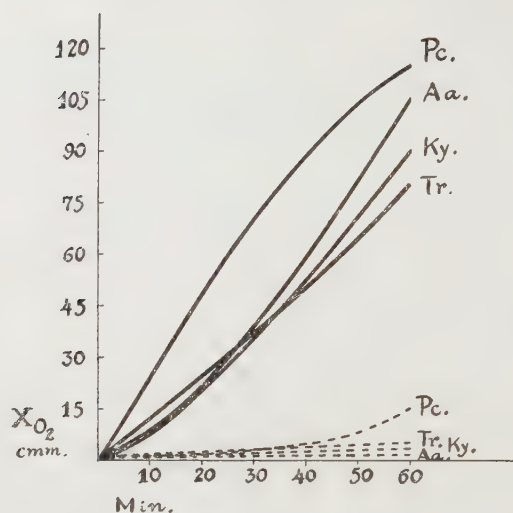


Fig. 1. Comparison of oxygen consumptions measured for each intermediate metabolite with tryptophan-adapted (full lines) and non-adapted cell (dotted lines).

Various substances which might be expected to come next in the oxidative breakdown of anthranilic acid were added as substrates to cell suspensions which had been previously adapted to *L*-tryptophan, and the oxygen consumption measured for each substrate. The total oxygen consumption for the complete dissimilation of anthranilic acid was 7–10 atoms, showing some divergency from the theoretical value.

The following substances were not oxidized:

orthoaminophenol	5-hydroxyanthranilic acid	salicylic acid
hydroquinon	3-hydroxyanthranilic acid	3-hydroxysalicylic acid
phenol	benzoic acid	p-amino benzoic acid
		p-amino phenol

5-Hydroxy-anthranilic acid was found by Professor Kotake in the urine of rabbits to which were injected anthranilic acid. This was synthesized also by Professor Kotake and is known for its biochemical effectiveness in very high dilutions. One of us, have previously reported (4) that 5-hydroxy-anthranilic acid undergoes oxidation reduc-

tion reaction between lactic acid dehydrogenase and laccase, or cytochrome C-cytochromoxidase system. In the experiment with our adapted cells, however, 5-hydroxy-anthranilic acid as well as 3-hydroxy-anthranilic acid could not be supposed to be the intermediate products in the oxidative breakdown of anthranilic acid.

Pyrocatechin alone, was proved to be active for our adapted cells. This relation is shown Fig. 1 by the curve Pc.. In the suspension of cells in which the adaptive enzyme system is fully developed, the rate of reaction increases with substance of lower metabolic order. This could be observed in the four curves Tr, Ky, Aa and Pc in Fig. 1. When 0.2 cc of the M/100 solution of *L*-tryptophan, *L*-kynurenin, anthranilic acid, or pyrocatechin are separately added to the non-adapted cells (washed cell suspensions harvested from the basic media), and the times required for the appearance of active oxygen consumption brought about by the formation of adaptive enzymes for the oxidation of the respective substrates are measured, *L*-tryptophan was found to require 5-6 hours, *L*-Kynurenin 4-5 hours, anthranilic acid 3-4 hours and pyrocatechin 1 hour. Thus, adaptation takes place more readily with substance of lower metabolic order. Further more, we have confirmed that the adaptive enzymes are produced or activated by the mere presence of the substrates without the need of cell multiplication.

From the foregoing results we have postulated that *L*-tryptophan is dissimilated by our strain of *Pseudomonas* via *L*-kynurenin, anthranilic acid and pyrocatechin, leading to the rupture of the benzene nucleus.

An example of complete dissimilation of anthranilic acid and pyrocatechin in the *L*-tryptophan adapted cells is given in Fig. 2. The difference of the total oxygen consumption of the two substrates corresponds well with the theoretical value of 1 atom of oxygen. Carbon dioxide output of the two substrates compared by measuring the respiration quotient showed within the limits of experimental error, a difference of 1 molecule of CO_2 : Thus, we have postulated

this reaction as follows:

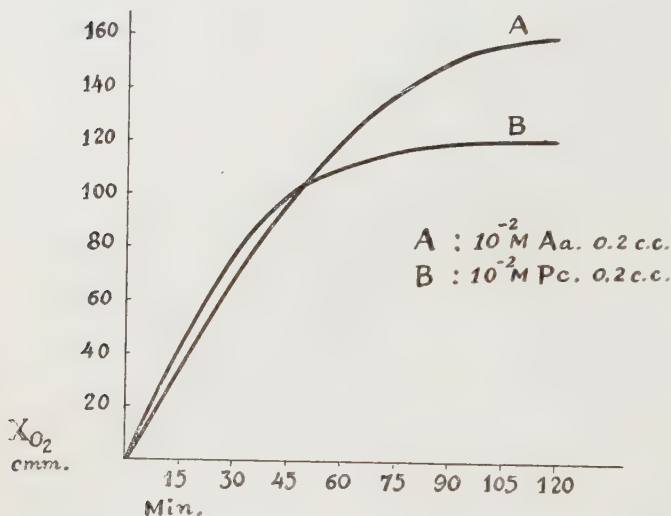
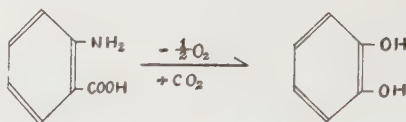


Fig. 2. Complete oxidative breakdown of anthranilic acid (Aa) and pyrocatechin (Pc) in tryptophan-adapted cell.

The foregoing facts were analysed further by the following experiments. To every ml. of washed suspension of cells harvested from the basic medium was added 1.0mg. of *L*-tryptophan, and equal volume of fresh nutrient broth added as source of nitrogen to permit cell multiplication. This was shaken at 30°. From this the portions were taken after one, three and five hours shaking centrifuged, washed and resuspended in *M*/15 phosphate buffer of pH, 7.2, and the concentration of the cells equalized. To these were added as substrates 0.2 ml. each of *M*/100 solution of *L*-tryptophane, *L*-kynurenin, anthranilic acid and pyrocatechin, and oxidative breakdown of each substrate compared. By this method the clarification of the mode of activation

of the enzyme systems corresponding to each of these four substrates was anticipated. The result of this experiment is shown in Fig. 3. As it is apparent here, up to 5 hours the enzymes of tryptophan, kynurenin and anthranilic acid are activated in the metabolic order. After 5 hours the mode of activation is not clear. When, however,

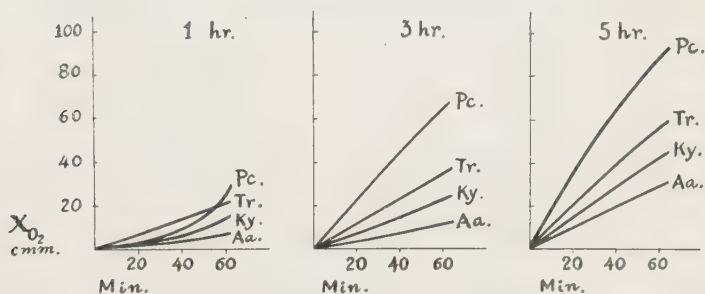


Fig. 3. Analysis of enzymatic adaptation to each intermediate metabolite when tryptophan is added as substrate in shaking culture.

this is compared with the results shown in Fig. 1, in which the cells were taken after 15 hours of shaking, in consideration of the fact that adaptation takes place more readily with substance of lower metabolic order, it is probable that the activity of these enzyme systems reverse after the lapse of 5 hours. It is note worthy that pyrocatechin-oxidase is not fully activated in the one hour adapted cells; however, in the three hours adapted cells a marked activation of this enzyme is observable. This may probably be due to the insufficient breakdown of tryptophan during the one hour period, in consequence of which the accumulation of pyrocatechin necessary for the activation of pyrocatechin-oxidase is not brought about in the cells. Thus, the activity of adaptive enzyme appear to surge along like the crest of sea waves from substance of higher metabolic order to that of the lower.

Varying amounts of anthranilic acid were added to the basic medium in which was cultured for 15 hours our strain of *Pseudomonas*. A definite amount of cells was collected and washed and

cell suspension made as already described. To these were added 0.2 ml. of $M/100$ solution of pyrocatechin and the activity of pyrocatechin-oxidase compared. As it is apparent in Fig. 4, the activity of the enzyme was found to be in equal proportion to the amount of anthranilic acid added to the basic medium.

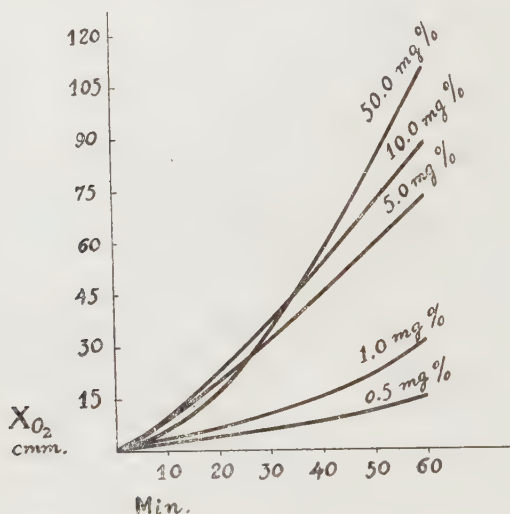


Fig. 4. Comparison of activity for pyrocatechin-oxidase with various amounts of anthranilic acid added to the basic medium.

The adaptive enzymes as will be mentioned later, are readily inactivated in the optimal temperature of growth in the absence of the substrate or source of substrate. Even when this fact is taken into consideration, the curves in Fig. 4 are indicative of the metabolic order of anthranilic acid and pyrocatechin. Conversely, cells adapted to pyrocatechin do not dissimilate anthranilic acid.

The pyrocatechin concentration and the corresponding activation of adaptive enzyme was examined next. To a definite amount of washed cell suspensions harvested from the basic medium was added pyrocatechin in varying amounts of $M/100$ to $M/12800$ (end concentration) as shown in Fig. 5. After 70 minutes of incubation at 30° ,

the cells were collected and washed by centrifugation to remove the substrate and the concentration of each cell suspension equalized. To these were added anew, 0.2 ml. of $M/100$ solution of pyrocatechin as substrate, and oxygen consumed in 10 minutes measured for each sample. As it is apparent in Fig. 5, the optimal coconcentration of pyrocatechin for the activation of adaptive enzyme is around $M/1000$.

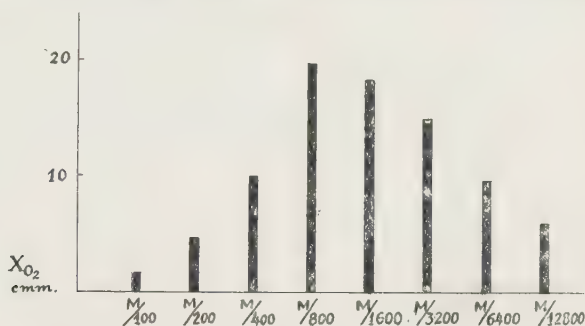


Fig. 5. Optimal concentration of pyrocatechin for the formation of adaptive enzyme.

Further more, the adaptive enzyme when once activated easily breaks down the substrate of such high concentration as $M/50$; that is, there is no inhibition of enzymatic activity due to over-concentration of substrate. Therefore, this optimal concentration of the substrate could be considered as the necessary condition for the adaptive process to proceed within the living cells. This may also be the optimal concentration of the pre-enzymatic substance within the cells of which will be dealt with later on.

When the cells were dried with acetone and a suspension made with the dry powder obtained, it displayed activity only towards pyrocatechin. Freezing and thawing process yielded a substance utterly inactive. When the cells were destroyed by ultra sonic waves and the cell body removed by centrifugation at 12000 r. p. m., the cell free supernatant fluid displayed powerful activity only towards pyrocatechin. In Fig. 6 is shown the oxygen uptake of 0.3 ml. of $10^{-2} M$

solution of pyrocatechin and 1.0 ml. of enzymatic fluid prepared in the following manner: cells harvested from 15 hours culture in the medium containing 1.0 mg. of anthranilic acid per ml. were exposed to ultrasonic waves for 15 minutes and the cell body removed by high speed centrifugation. The cell free fluid prepared in the similar manner from cells grown in the basic medium alone did not contain pyrocatechin-oxidase.

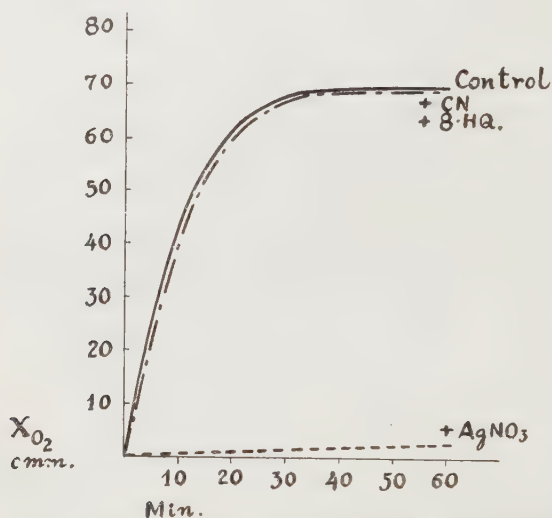


Fig. 6. Oxygen consumption for pyrocatechin by cell-free enzyme (full-line) and inhibition for the activity of this enzyme by $10^{-3} M$ KCN, $10^{-3} M$ 8-hydroxy-quinoline, and $10^{-4} M$ $AgNO_3$. (dotted lines.)

The enzymatic action of pyrocatechin-oxidase consumed 2 atoms of oxygen per mole of pyrocatechin, and carbon dioxide output was not detectable. Neither KCN nor 8-hydroxy-quinoline inhibited the activity of this enzymatic fluid. The rupture of the benzene ring during the oxidation process was confirmed through the absence of benzene nucleus by spectrophotometry, and by the negative phenolic reaction with Folin-Ciocalteu's reagent, after the termination of the reaction.

Since Jaffe's report in 1909 that urine of dogs fed with benzene

yield muconic acid, the rupture of the benzene nucleus were reported only *in vivo*. We have however, succeeded in obtaining this enzyme in a cell free state. The purification of this enzyme with ammonium sulfate is now progressing. As it is shown in Fig. 6. marked inhibition is caused by $10^{-4} M$ solution of $AgNO_3$, and so we are of the opinion that this enzyme might be a flavinenzyme. The end product of this reaction would probably be defined by the purified enzyme.

In the foregoing experiments we have shown that adaptive process of the enzymatic system in the oxidative breakdown of tryptophan proceed "successively", and from the standpoint of "successive adaptation" have found a new enzymatic system in substance of lower metabolic order.

We extended our experiment in the following manner to clarify some points concerning the nature of bacterial adaptation. We have already shown that the adaptive enzymes are produced without the source of nitrogen, that is, with substrate and buffer alone. In order to carry out our experiment with cells in a complete static condition, we employed bacteriostatic substances to inhibit cell proliferation. Unlike Mirick's strain, which was sensitive to the sulfa drugs, our strain was not influenced neither by the sulfa drugs nor by penicillin. Streptomycin, however, inhibited growth as shown below.

Suspensions were prepared from cells harvested from the basic medium to which was added streptomycin in the following dilutions and with $10^{-2} M$ of pyrocatechin the adaptation curves were traced as shown in the right side curves in Fig. 7. Unexpectedly, the formation of the adaptive enzyme was found to be inhibited in direct proportion to the concentration of streptomycin. However, 10 times

Antibacterial potency of streptomycin to *Pseudomonas* sp. used.

1/Mol.	10^4	2×10^4	4×10^4	8×10^4	1.6×10^5	3.2×10^5	Control
rate of cell							
growth.	—	—	—	+	++	+++	+++

the amount of streptomycin capable of inhibiting severely the formation of the adaptive enzyme ($M/2 \times 10^4$) had no effect upon the cells

which had been well adapted to pyrocatechin. (left side curve in Fig. 7.). This indicates that streptomycin has no effect on the enzyme system itself. In this case the absence of nitrogen supply would not permit the cells to multiply, or even if they did it would be only in some immeasurable degree. In spite of it the inhibitory action of streptomycin is indicative that this antibiotics inhibits the activation

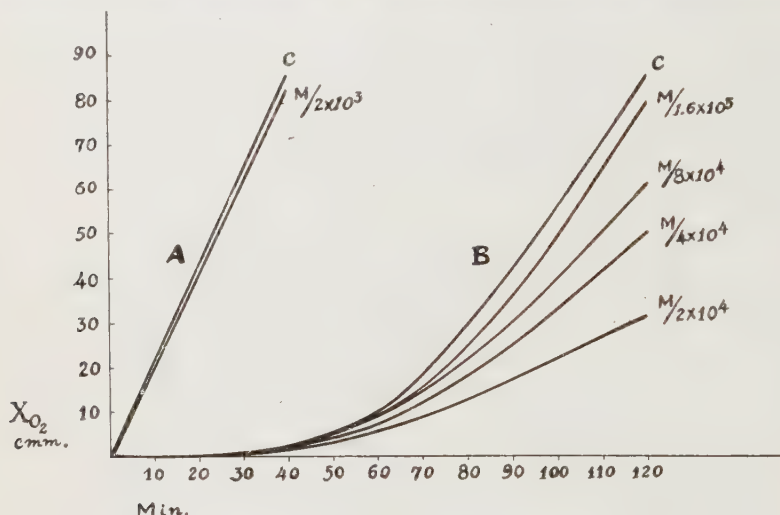


Fig. 7. Effects of streptomycin on adapted and non-adapted cells.

A; adapted cells. B; non-adapted cells. C; without streptomycin.

Substrate; $10^{-2} M$ pyrocatechin 0.2 ml. in each case.

or synthesis of the adaptive enzyme within living cells. At this point there arise a necessity to postulate a highly reactable pre-enzymatic substance existing within the cell. We would not like to draw a hasty conclusion from these facts concerning the mechanism of the action of antibiotics. Nevertheless, action against this pre-enzymatic substance within the cells may be one of the mechanisms of inhibition of bacterial growth. The existence of an optimal concentration of the substrate towards the pre-enzymatic substance as already mentioned, seems to indicate that in contact with the substrate the enzymatic system begins to develop autocatalytically.

The stability of the adaptive enzymes and the interaction between adaptive enzymes were next experimented.

As it is shown in Fig. 8, inactivation of different enzyme systems in the oxidative breakdown of *L*-tryptophan is relatively slow when the washed cells without substrates are allowed to stand in the refrigerator. After 48 hours more than 60% of the activity is retained. When however, the same suspension is shaken at 30°, a rapid loss of activity is seen in 1-2 hours, as indicated by the top line in the right side of Fig. 8. This is an example with cell suspension adapted to *L*-tryptophan, so in comparison with the curve Aa on the left where the activity is retained 80% after 48 hours, only 65% is retained here after two hours.

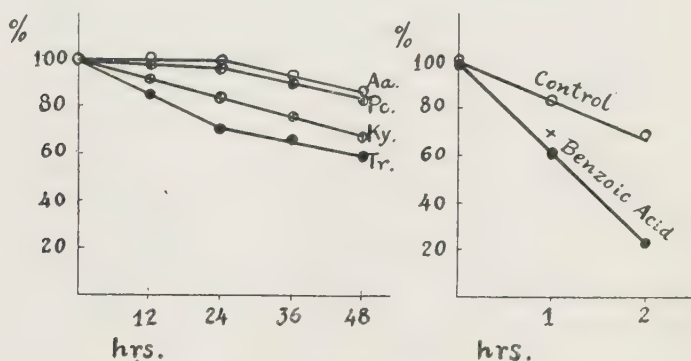


Fig. 8. The stability of adaptive enzymes and the influence of other substrates on the enzyme system ready formed.

The left side: Loss of enzyme activities in *L*-tryptophan adapted cells stored in ice-box; *L*-tryptophan (Tr.), *L*-kynurenin (Ky.), Anthranilic acid (Aa.) and pyrocatechin (Pc.) added as substrates.

The right side: Loss of enzyme activities in the same cells shaking at 30° in the presence and the absence of benzoic acid; Aa added as substrate.

Another interesting point is that though this strain also breaks down benzoic acid, when benzoic acid is added anew during shaking at 30° the loss of activity of the oxidative enzyme of anthranilic acid is speeded considerably. This is shown on the right hand side

of Fig. 8. This indicates that there exists an interaction between the enzyme systems of benzoic acid and anthranilic acid. The interaction of galactozymase and glucozymase in yeast has been reported in details by Stephenson and Yudkin, and further confirmed by Spiegelman. It could be said that in cells adapted to a definite substrate, the activity of a similar reaction system is generally inhibited. This fact is one of the basis on which is grounded the effectiveness of the method of tracing the dissimilatory process by means of the adaptive enzymes.

Furthsr more, Fig. 9 shows that cells adapted to anthranilic acid adapt quicker to benzoic acid than the non-adapted cells. This fact with that of Fig. 8, indicates that under the influence of the respective substrates, the reaction (adaptive enzyme of anthranilic acid $\rightleftharpoons$ adaptive enzyme of benzoic acid) is taking place in the pre-enzymatic substance within the cells.

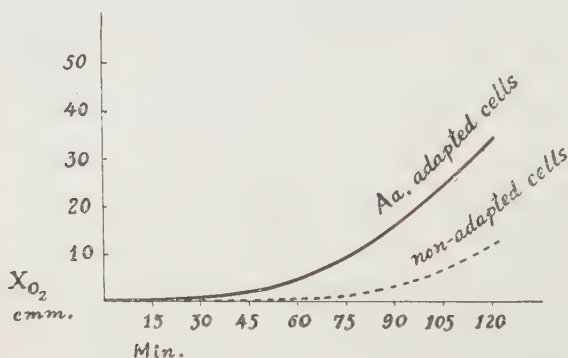


Fig. 9. Comparision of enzymatic adaptation to benzoic acid with anthranilic acid adapted and non-adapted cells.

Further experiment in this line is now under way, and we expect to make another rsport in the near future.

Conclusions :

1. We have analysed the enzymatic systems involved in the oxidative breakdown of tryptophan by using a strain of *Pseudonomas*

sp. and have found that the speed of adaptation and the velocity of reaction gradually increase with substance of lower metabolic order, and have named this phenomena of enzyme adaptation "successive adaptation".

2. We have established a biochemical method of tracing unknown metabolic routes by the use of this phenomena of successive adaptation. This method is available as well as isotopes for tracing metabolic pathway. This method is grounded on the following facts:

a). When the cells are adapted to a substance of higher metabolic order, the adaptive enzymes for substances that come in the metabolic pathway below it are also produced specifically.

b). Conversely, when the cells are adapted to substance of lower metabolic order, the enzymes for substances of higher order are not produced.

c). The enzymes for substances of lower metabolic order are produced in proportion to the concentration of the substance of higher order to which the cells are adapted.

d). The enzyme systems partaking in the route of breakdown of any substance are activated by genetically controlled biochemical reaction peculiar to the microorganism.

3. We have successively isolated in a cell free state an enzyme which reapture the benzene nucleus of pyrocatechin, and have named it pyrocatecase.

4. We have found that in the formation of adaptive enzymes, a highly reactable pre-enzymatic substance must be postulated to exist within the cells. Each substrate has its own optimal concentration towards this pre-enzymatic substance, and the bacteriostatic action of streptomycin against our strain of *Pseudomonas*, appears to be the result of some action dealt upon this pre-enzymatic substance.

5. We have experimented and made discussions on the interaction between different adaptive enzymes.

We would like to express our gratitude for the valuable advice given us by Professor Akabori (Department of science, Osaka University.) and Assistant Professor Amano of the bacteriological section and also thank Professor Kotake and Professor Ichihara for their kindness in granting us valuable samples of kynurenin, hydroxy-anthranilic acid and tryptophan.

REFERENCES.

- (1) Spiegelman S., Reiner J.M. & Cohnberg R., *J. Gen. Physiol.*, **31**, 27 (1947); Reiner J. M. & Spiegelman S., *Ibid*, **31**, 58 (1947); Spiegelman S., Dunn R., *Ibid*, **31**, 153 (1947); Spiegelman S., Reiner J. M., *Ibid*, **31**, 175 (1947).
- (2) Mirick G.S., *J. Exp. Med.*, **78**, 255 (1943).
- (3) Stanier R.Y., *J. Bact.*, **54**, 339 (1947).
- (4) Suda M., announced at the Annual Meeting of the Society of Japanese Biochemists in April 1947 at Osaka.

PYROCATECASE

A NEW ENZYME CATALIZING OXIDATIVE BREAKDOWN OF PYROCATECHIN.*

By

OSAMU HAYAISHI and KIZO HASHIMOTO.

*(Department of Bacteriochemistry, Research Institute for Microbial
Diseases, Osaka University, Osaka)*

(Received for publication June 7, 1950)

As reported (1) previously, we obtained cell free enzyme from a strain of *Pseudomonas* sp. adapted to anthranilic acid or tryptophan, which catalyzed the oxidative breakdown of pyrocatechin with the rupture of benzene nucleus. We named this enzyme "pyrocatecase."

The present communication is concerned with further purification of this enzyme and mode of its actions.

EXPERIMENTAL

Material:

To obtain the adapted cells 100 to 200 mg. per cent of specific substances such as L-tryptophan or anthranilic acid are added to the ordinary pepton-broth. The cells are harvested from 15 hours culture at 30°, centrifuged and washed twice with distilled water and then dried with acetone.

Purification of the Enzyme:

The activity of enzyme is defined as $Q_{O_2/N}$, the oxygen consumption per hour per Kjeldahl nitrogen for 0.2 ml. of *M*/10 pyrocatechin. To simplify the explanation, we will illustrate the course of enzyme preparation by the following table.

*The compendium of this article was announced at the Annual Meeting of the Society of Japanese Biochemists in April 1949 at Kyoto University and at the Symposium on Enzyme Chemistry in October 1949 at Tokyo University.

TABLE I

1 g. of acetone dried cells ($Q_{O_2}/N=248$) was suspended in 30 ml. *M/15* phosphate buffer (pH 8.0), incubated for 48 hours in ice box, then centrifuged at 15,000. r.p.m. for 20 minutes.

Supernatant (reddish brown)
fractionated with 0.3-0.4
saturated $(NH_4)_2SO_4$

Residue discarded.

Supernatant

Residue dissolved in *M/15* phosphate buffer (pH 8.0). To remove impurities, $Ca_3(PO_4)_2$ gel added (25 vol. %), then centrifuged at 3,000. r.p.m. for 15 minutes.

Supernatant ($Q_{O_2}/N=745$)
fractionated with 0.3-0.5
saturated $(NH_4)_2SO_4$

Residue discarded

Supernatant discarded

Residue dissolved in *M/15* phosphate buffer (pH 8.0). To remove impurities kaolin was added, then centrifuged at 3,000. r.p.m. for 15 minutes.

Supernatant ($Q_{O_2}/N=2015$)
fractionated with 0.3-0.4
saturated $(NH_4)_2SO_4$

Residue discarded

Supernatant discarded

Sediment dissolved in *M/15* phosphate buffer (pH 8.0), dialyzed overnight at room temperature against dilute NH_3 solution. Internal fluid adjust to pH 7.2. To adsorb the enzyme, Cr was added (30 vol. %). The suspension was incubated at room temperature for 4 hours with occasional shaking, then centrifuged.

Supernatant discarded

Residue eluted overnight in ice box with *M/15* phosphate buffer (pH 8.2). The eluate is fractionated with 0.3-0.4 saturated $(NH_4)_2SO_4$

Supernatant discarded

Residue dissolved in *M/15* phosphate buffer at pH 6.0, made up to a definite volume.

We obtained thus colorless and transparent enzyme solution, the activity of which was calculated to be $Q_{O_2}/N=10,600$.

Some Properties of The Enzyme :

The pH optimum is 7.0-7.4. When the pH is made more acid than the optimum value, the enzyme loses its activity very rapidly. Accordingly in the course of enzyme preparation, we must avoid the shifting of pH towards acid side. O_2 uptake measured by Warburg's technique is 2 atoms of oxygen per mole of substrate, but no CO_2 release was observed. The enzyme activity is inhibited completely with $10^{-3} M$ $AgNO_3$, but partially, with $10^{-3} M$ $CuSO_4$. No inhibition was found with the following reagents: KCN, NaN_3 , NaF, 8-hydroxyquinoline, semicarbazide, aniline, and CO.

The purified enzyme is colorless and we cannot prove FAD present in this preparation by Alanine-test.

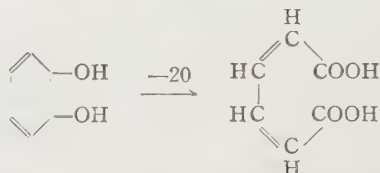
The End Product of Enzyme Reaction :

60 ml. of the purified enzyme solution and 250 ml. of pyrocatechin in 10 ml $M/15$ phosphate buffer (pH 7.4) are incubated in shaking flask at 38° for 3 hours. To avoid shifting of pH of the liquid towards acid side, diluted NaOH solution was pipetted in at intervals of 10 minutes. The rate of reaction is checked with Folin's reagent. After the phenolic reaction in the small sample of fluid has decreased to a minimum value, stopped the reaction with conc. H_2SO_4 to bring the pH of the fluid to 2.8. After heating the fluid at 70° for 15 minutes, centrifuged, the supernatant extracted with ethyl ether by the Kutscher-Steudel apparatus. Ether is reextracted twice with saturated $NaHCO_3$ solution. The combined extracts are again adjusted to pH 3.0 with conc. H_2SO_4 and then extracted with ethyl ether in separating funnel, and the ether is distilled off under reduced pressure. The light yellow powder was obtained as a residue. After washing the powder with a small amount of distilled water, it is dissolved in ethyl ether and ether is evaporated slowly at room temperature under reduced pressure. Fine needles appear. The dry weight of crystalline substance is about 125 mg., melting point 181° . Analytical value; C 50.88%, H 4.37%.

It is most likely that this substance is *cis-cis* muconic acid.

The Nature of Enzyme Action:

From the above data, it is suggested that the enzyme "pyrocatecase" catalyses the following reaction:



Dialyzing the enzyme for 48 hours against distilled water in ice box causes no influence on its activity. Inorganic phosphate or ATP does not participate in the reaction. As, in the course of enzyme action, the fluid has not been colored and $M/1000$ aniline has no effect on the reaction, it is most unlikely that any quinone-substances appeared as intermediates.

It is suggested that both the rupture of benzene nucleus and oxidation of the substrate occurred simultaneously in this reaction.

SUMMARY

1. From the acetone powder of a strain of *Pseudomonas* sp., adapted to anthranilic acid or tryptophan, a cell free enzyme has been isolated and fairly purified.

2. The enzyme, named pyrocatecase, is inhibited with AgNO_3 and CuSO_4 , but not with other general inhibitors.

3. *Cis-cis* muconic acid has been proved as the endproduct of the enzyme action.

4. From the data obtained, it appears for us to be most probable that tryptophan, kynurenine, and anthranilic acid are metabolized via pyrocatechin and muconic acid in a *Pseudomonas* sp.

We wish to express our gratitude to the head of this laboratory, Assist. Prof. Dr. M. Suda, for his careful advice and kind support.

REFERENCES.

- (1) Suda, M. Hayaishi, O. Oda, & Y. Medical Journal of Osaka University. (in press); J. Biochem., **37**, 355 (1950).

METABOLISM OF TYROSINE

1. APPLICATION OF SUCCESSIVE ADAPTATION OF BACTERIA FOR THE ANALYSIS OF THE ENZYMATIC BREAKDOWN OF TYROSINE.*

By

MASAMI SUDA and YOSHIRO TAKEDA

(Department of Bacteriochemistry, Research Institute for Microbial Diseases, Osaka University, Osaka)

(Received for publication June 7, 1959)

In 1941, Felix *et al.* (1) demonstrated the oxidative breakdown of tyrosine with homogenate of rabbit liver. From the amount of oxygen consumption, they concluded that homogentisic acid did not account for an intermediate in the course of tyrosine metabolism.

We took up this problem from the viewpoint of our method of successive adaptation (2) for the analysis of metabolic pathway of tyrosine. It was proved from our data that the homogentisic acid may be an intermediate. From the tyrosine adapted cells, we have prepared enzyme "homogentisicase", catalyzing the oxidative breakdown of homogentisic acid with the rupture of its benzene ring.

EXPERIMENTAL.

Materials:

A strain of *Pseudomonas sp.*, having the capacity to dissimilate tyrosine, was isolated from soil. The cells are harvested from 24 hours culture at 30° in inorganic media (KH₂PO₄ 1.5g., (NH₄)₃ PO₄ 2.5g., glucose 5.0g., MgSO₄ 0.1g., NaCl 5g., water 1000 ml.), washed twice with distilled water and resuspended in M/15 phosphate buffer (pH 7.4). This suspension, called the normal cell, is further adapted to 10⁻³ M L-tyrosine shaking at 30° for 2 hours. The adapted cells are collected, washed as above by centrifugation.

*The compendium of this article was announced at the Annual Meeting of the Society of Japanese Biochemists in April 1949 at Kyoto University and at the Symposium on Enzyme Chemistry in October 1949 at Tokyo university.

Pb-salt of homogentisic acid was isolated from urine of alkaptonurica and purified as free acid.

Results:

The suspensions of the bacteria are made with the normal and adapted cells, and the density equalized. 0.2 ml. of *M*/100 substrate and 2 ml of bacterial suspensions are separately placed in the side arm and in the main compartment of Warburg vessels respectively. O_2 uptake is measured as usual.

As shown in Fig. 1 the normal cells cannot dissimilate L-tyrosine and homogentisic acid so rapidly at the adapted cells. When the normal cells are adapted to homogentisic acid, the enzyme system related to this substance and its successive metabolites are formed, while activity for tyrosine is hardly measurable. Cells adapted to L-tyrosine cannot metabolize the following substances; parahydroxyphenyl pyruvic, lactic, and acetic acids, gentisic acid, benzoic acid, parahydroxybenzoic acid, dopa, L-phenylalanine, formyl-L-tyrosine,

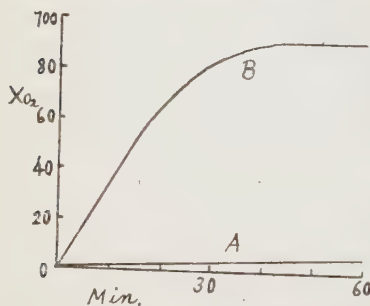


Fig. 1. Comparison of the rates of O_2 uptake with (a) tyrosine-adapted and (b) unadapted cells.

A. Substrate: 0.2 ml of *M*/300 tyrosine.

B. Substrate: 0.2 ml of *M*/100 homogentisic acid. Temperature 30° .

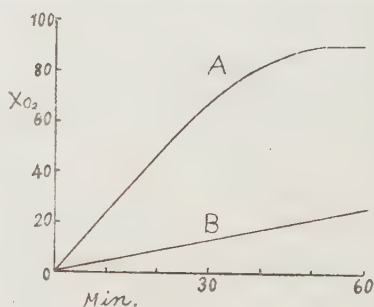


Fig. 2. Comparison of the rates of O_2 uptake of homogentisic acid (HGA) with acetone powder of tyrosine adapted (A) and unadapted cells (B).

Substrate: 0.2 ml of *M*/50 HGA. Temperature 30° .

hydroquinone, pyrocatechin. Cells adapted to L-phenylalanine can oxidize L-tyrosine and homogentisic acid as rapidly as the tyrosine adapted cells.

We demonstrated the homogentisic acid oxidase in the acetone preparation of the adaptive cells. The powders are suspended in *M*/15 phosphate buffer (pH 7.4), containing 5 mg. per ml. 2 ml of this suspension are placed in Warburg flask, 0.2 ml. of *M*/50 homogentisic acid (adjusted to pH 7.4) in side arm, and the oxygen consumption was determined as usual. The amount of oxygen taken up per mole of the substrate is calculated from Fig. 2 to be 2 atoms, no CO_2 release being measured. The acetone powder of normal cells did not possess the activity to oxidize homogentisic acid.

During the manometric determination, the liquid has not been colored. After the reaction has terminated a small sample of liquid is pipetted out, and checked with Folin's reagent. Any phenolic substances could not be detected. It is suggested that the benzene ring of homogentisic acid may be ruptured. We confirmed this subsequently as it will be reported in the next paper of this series.

We succeeded in a preparation of cell free enzyme from the acetone powder as follows: 1 g. of the powder of the adapted cells is suspended in 30 ml of *M*/10 phosphate buffer (pH 7.2), alternatively frozen and thawed rapidly 8 times, added with the same buffer to give 50 ml. volume, and centrifuged. The supernatant liquid contains most of the original activity.

DISCUSSION

Using our successive adaptation method, we have recognized that homogentisic acid is an intermediate of tyrosine metabolism in *Pseudomonas* *sp.* However, *p*-hydroxyphenylpyruvic acid is not broken down by the tyrosine adapted cell. Recently Neuberger (3) synthesized 2,5-dihydroxyphenylalanine as a probable intermediate in the breakdown of tyrosine and he reported that this substance was metabolized by animal body.

From these experiments, it is conceivable that tyrosine may be metabolized *via* 2,5-dihydroxyphenylalanine to homogentisic acid and not *via* *p*-hydroxyphenylpyruvic acid.

As reported above, we succeeded the isolation of cell-free enzyme from tyrosine adapted cells, catalyzing the oxidative breakdown of homogentisic acid. Therefore, contrary to the Felix's report, it is reasonable that homogentisic acid is a normal metabolite in the breakdown of tyrosine.

SUMMARY

1. Washed suspension of a strain of *Pseudomonas* sp. adapted to L-tyrosine acquires an activity to dissimilate L-tyrosine and homogentisic acid. Cells adapted to the latter can not oxidize the former. The unadapted cells are unable to oxidize any of the above substrates so rapidly. Possible intermediates such as parahydroxyphenylpyruvic acid, gentisic acid, and others as described above cannot be metabolized by the tyrosine-adapted cells.

2. The acetone powder of tyrosine-adapted cells possesses an activity to oxidize homogentisic acid, while the same preparation from unadapted cells has none.

3. It is most likely that L-tyrosine is metabolized via homogentisic acid.

4. From the adapted cells, we have prepared a cell free enzyme "homogentisicase", catalyzing the oxidative breakdown of homogentisic acid with rupture of benzene ring. 2 atoms of oxygen per mole of substrate are determined, and no CO_2 release can be measured.

REFERENCES

- (1) Felix, K., & Zorn, K., *Z. Physiol. Chem.*, **268**, 257, (1941)
- (2) Suda, M., Hayaishi, O., & Oda, Y., *Medical Journal of Osaka University*. (in press); *J. Biochem.*, **37**, 355, (1950)
- (3) Neuberger, A., *Biochem. J.*, **43**, 599, (1948)

CRYSTALLOGICAL RESEARCH ON EMMENIN AND OESTEROL WITH THE POLARIZING MICROSCOPE (RESUME)

By

YAEKO KAWAI

Director ; Prof. Kentaro Hayakawa M.D.

Dept. of Physiological Chemistry, Dental College Nihon University.

(Received for publication, June 7, 1950)

Wiesner and Marshall (1) disagreed with the report of Collip *et al.* (2) who extracted emmenin from fresh placentas. Later, Butenandt and Browne (3), through their researches, state that it is probably the same substance as oesteriol. But Collip himself showed that because the emmenin found in the placenta is saponified by alkalis, it is probably an ester form and also because it is insoluble in other, it is difficult to say that it is the same substance as oesteriol. Oesteriol is probably the mother-substance.

The author, in trying to solve this question, first extracted the so-called emmein from placenta according to Collip's method and then extracted oesteriol from pregnant women's urine according to Janney and Walker's method (4) and obtained the crystals with a polarizing microscope.

The extracted substance which was considered to be emmenin was recrystallized with alcohol. It was found not to be double refracting, but a hexahedron or a cubic crystal. Further, when heated on a platinum plate, it was carbonized immediately and underwent combustion without leaving any ash. The melting point is 182°.

The obtained oesteriol crystals appeared to be the same as those extracted by Doisy (5) and belong to the rhombic crystal system as a result of the author's observations. The parameter ratio is

$b:c = 1:1.083$, negative, double refraction plane of optical axis b 010, axial angle $V-36^{\circ}43'$ (Berek's method (6), the appearance of crystals usually shows a (100) plane extending to C axis. The apparent axial obtuse angle by oil immersion is approximately 59° on this plane.

As a result of the above-mentioned findings, the author concludes that emmenin and oesteriol are entirely different substances, agreeing with Collip's postulation. But, at the same time, it can not be excluded that oesteriol is probably formed by the decomposition of emmenin.

The author is continuing research on this subject.

REFERENCES

- (1) Wiesner, B. P. & Marshall. *Chem., Zentralb.* 1, 404 (1932)
- (2) Collip, J. B., Thomson, D. L., Browns, J. S. L. McPhail, M. K., & Williamson, J. E., *Endocrinol*, 15, 315 (1931)
- (3) Butenandt, A., & Brawne, J. S. L. Z., *physiol. Chem.*, 216, 49 (1933)
- (4) Janney, T. C., & Walker, B. S., *Endocrinol*, 14, 101 (1930)
- (5) Doisy, E. A., Veler, C. D., & Thaayer, S., *J. Biol. Chem.* 86, 499 (1930)
- (6) Berek, M., *Neue Wege Zur Universalmethode; Mitteilungen der Leitz Werke*, Nr. 27, (1923)